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(54) **Title:** HUMAN KALLIKREIN-LIKE GENES

(57) **Abstract:** The invention relates to nucleic acid molecules, kallikrein-like proteins encoded by such nucleic acid molecules; and use of the proteins and nucleic acid molecules

**TITLE: Novel Human Kallikrein-Like Genes****FIELD OF THE INVENTION**

5 The invention relates to nucleic acid molecules, proteins encoded by such nucleic acid molecules; and use of the proteins and nucleic acid molecules

**BACKGROUND OF THE INVENTION**

Kallikreins and kallikrein-like proteins are a subgroup of the serine protease enzyme family and exhibit a high degree of substrate specificity (1). The biological role of these kallikreins is the selective cleavage of specific polypeptide precursors (substrates) to release peptides with potent biological activity (2). In mouse and rat, kallikreins are encoded by large multigene families. In the mouse genome, at least 10 24 genes have been identified (3). Expression of 11 of these genes has been confirmed; the rest are presumed to be pseudogenes (4). A similar family of 15-20 kallikreins has been found in the rat genome (5) where at least 4 of these are known to be expressed (6).

Three human kallikrein genes have been described, i.e. prostatic specific antigen (PSA or KLK3) (7), human glandular kallikrein (KLK2) (8) and tissue (pancreatic-renal) kallikrein (KLK1) (9). The PSA gene spans 5.8 Kb of sequence which has been published (7); the KLK2 gene has a size of 5.2 Kb and its complete structure has also been elucidated (8). The KLK1 gene is approximately 4.5 Kb long and the exon sequences and the exon/intron junctions of this gene have been determined (9).

The mouse kallikrein genes are clustered in groups of up to 11 genes on chromosome 7 and the distance between the genes in the various clusters can be as small as 3-7 Kb (3). All three human kallikrein genes have been assigned to chromosome 19q13.2 – 19q13.4 and the distance between PSA and KLK2 has been estimated to be 12 Kb (9).

A major difference between mouse and human kallikreins is that two of the human kallikreins (KLK2 and KLK3) are expressed almost exclusively in the prostate while in animals none of the kallikreins is localized in this organ. Other candidate new members of the human kallikrein gene family include protease M (10) (also named Zyme (11) or neurosin (12) and the normal epithelial cell-specific gene-1 (NES1) (13). Both genes have been assigned to chromosome 19q13.3 (10,14) and show structural homology with other serine proteases and the kallikrein gene family (10-14).

**SUMMARY OF THE INVENTION**

10 In efforts to precisely define the relative genomic location of PSA, KLK2, Zyme and NES1 genes, an area spanning approximately 300 Kb of contiguous sequence on human chromosome 19 (19q13.3 – q13.4) was examined. The present inventors were able to identify the relative location of the known kallikrein genes and, in addition, they identified other kallikrein-like genes which exhibit both location proximity and structural similarity with the known members of the human kallikrein family. The novel 15 genes exhibit homology with the currently known members of the kallikrein family and they are co-localized in the same genomic region. These new genes, like the already known kallikreins have utility in various cancers including those of the breast, testicular, and prostate.

The kallikrein-like proteins described herein are individually referred to as "KLK-L1, KLK-L2,

KLK-L3, KLK-L4, KLK-L5, or KLK-L6", and collectively as "kallikrein-like proteins" or "KLK-L Proteins". The genes encoding the proteins are referred to as "*klk-11, klk-12, klk-13, klk-14, klk-15, or klk-16*", and collectively as "kallikrein-like genes" or "*klk-l* genes".

Broadly stated the present invention relates to an isolated nucleic acid molecule which comprises:

- 5 (i) a nucleic acid sequence encoding a protein having substantial sequence identity with an amino acid sequence of KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively;
  - (ii) a nucleic acid sequence encoding a protein comprising an amino acid sequence of KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 as shown in SEQ.ID.NO. 2, 3, 10 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively;
  - (iii) nucleic acid sequences complementary to (i);
  - (iv) a degenerate form of a nucleic acid sequence of (i);
  - (v) a nucleic acid sequence capable of hybridizing under stringent conditions to a nucleic acid sequence in (i), (ii) or (iii);
  - 15 (vi) a nucleic acid sequence encoding a truncation, an analog, an allelic or species variation of a protein comprising an amino acid sequence of KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively; or
  - (vii) a fragment, or allelic or species variation of (i), (ii) or (iii).
- 20 Preferably, a purified and isolated nucleic acid molecule of the invention comprises:
- (i) a nucleic acid sequence comprising the sequence of SEQ.ID.NO. 1, 13, 21, 43, 56, or 65 wherein T can also be U;
  - (ii) nucleic acid sequences complementary to (i), preferably complementary to the full nucleic acid sequence of SEQ.ID.NO. 1, 13, 21, 43, 56, or 65;
  - 25 (iii) a nucleic acid capable of hybridizing under stringent conditions to a nucleic acid of (i) or (ii) and preferably having at least 18 nucleotides; or
  - (iv) a nucleic acid molecule differing from any of the nucleic acids of (i) to (iii) in codon sequences due to the degeneracy of the genetic code.

The invention also contemplates a nucleic acid molecule comprising a sequence encoding a truncation of a KLK-L protein, an analog, or a homolog of a KLK-L Protein or a truncation thereof. (KLK-L Proteins and truncations, analogs and homologs of KLK-L Proteins are also collectively referred to herein as "KLK-L Related Proteins").

The nucleic acid molecules of the invention may be inserted into an appropriate expression vector, i.e. a vector that contains the necessary elements for the transcription and translation of the inserted coding sequence. Accordingly, recombinant expression vectors adapted for transformation of a host cell may be constructed which comprise a nucleic acid molecule of the invention and one or more transcription and translation elements linked to the nucleic acid molecule.

The recombinant expression vector can be used to prepare transformed host cells expressing KLK-

L Related Proteins. Therefore, the invention further provides host cells containing a recombinant molecule of the invention. The invention also contemplates transgenic non-human mammals whose germ cells and somatic cells contain a recombinant molecule comprising a nucleic acid molecule of the invention, in particular one which encodes an analog of a KLK-L Protein, or a truncation of a KLK-L Protein.

5 The invention further provides a method for preparing KLK-L Related Proteins utilizing the purified and isolated nucleic acid molecules of the invention. In an embodiment a method for preparing a KLK-L Related Protein is provided comprising (a) transferring a recombinant expression vector of the invention into a host cell; (b) selecting transformed host cells from untransformed host cells; (c) culturing a selected transformed host cell under conditions which allow expression of the KLK-L Related Protein; 10 and (d) isolating the KLK-L Related Protein.

The invention further broadly contemplates an isolated KLK-L Protein comprising an amino acid sequence as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67.

The KLK-L Related Proteins of the invention may be conjugated with other molecules, such as proteins, to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins. 15

The invention further contemplates antibodies having specificity against an epitope of a KLK-L Related Protein of the invention. Antibodies may be labeled with a detectable substance and used to detect proteins of the invention in tissues and cells.

20 The invention also permits the construction of nucleotide probes which are unique to the nucleic acid molecules of the invention and/or to proteins of the invention. Therefore, the invention also relates to a probe comprising a nucleic acid sequence of the invention, or a nucleic acid sequence encoding a protein of the invention, or a part thereof. The probe may be labeled, for example, with a detectable substance and it may be used to select from a mixture of nucleotide sequences a nucleic acid molecule of the invention including nucleic acid molecules coding for a protein which displays one or more of the properties of a protein of the invention. 25

The invention still further provides a method for identifying a substance which binds to a protein of the invention comprising reacting the protein with at least one substance which potentially can bind with the protein, under conditions which permit the formation of complexes between the substance and protein and detecting binding. Binding may be detected by assaying for complexes, for free substance, or for non-complexed protein. The invention also contemplates methods for identifying substances that bind to other intracellular proteins that interact with a KLK-L Related Protein. Methods can also be utilized which identify compounds which bind to KLK-L gene regulatory sequences (e.g. promoter sequences). 30

Still further the invention provides a method for evaluating a compound for its ability to modulate the biological activity of a KLK-L Related Protein of the invention. For example a substance which inhibits or enhances the interaction of the protein and a substance which binds to the protein may be evaluated. In an embodiment, the method comprises providing a known concentration of a KLK-L Related Protein, with a substance which binds to the protein and a test compound under conditions which permit the formation of complexes between the substance and protein, and removing and/or detecting complexes. 35

Compounds which modulate the biological activity of a protein of the invention may also be identified using the methods of the invention by comparing the pattern and level of expression of the protein of the invention in tissues and cells, in the presence, and in the absence of the compounds.

The proteins of the invention and substances and compounds identified using the methods of the invention, and peptides of the invention may be used to modulate the biological activity of a KLK-L Related Protein of the invention, and they may be used in the treatment of conditions such as cancer (e.g. breast, testicular, and prostate cancer). Accordingly, the substances and compounds may be formulated into compositions for administration to individuals suffering from cancer.

Therefore, the present invention also relates to a composition comprising one or more of a protein of the invention, a peptide of the invention, or a substance or compound identified using the methods of the invention, and a pharmaceutically acceptable carrier, excipient or diluent. A method for treating or preventing cancer is also provided comprising administering to a patient in need thereof, a KLK-L Related Protein of the invention, or a composition of the invention.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

The invention will now be described in relation to the drawings in which:

Figure 1 shows an approximate 300 Kb of contiguous genomic sequence around chromosome 19q13.3 - q13.4 represented by 8 contigs, each one shown with its length in Kb. The contig numbers refer to those reported in the Lawrence Livermore National Laboratory website. Note the localization of the seven known genes (PSA, KLK2, Zyme, NES1, HSCCE, neuropsin and TLSP) (see abbreviations for full names of these genes). All genes are represented with arrows denoting the direction of transcription. The gene with no homology to human kallikreins is termed UG (unknown gene). The five new kallikrein-like genes (KLK-L1 to KLK-L5) were numbered from the most centromeric to the most telomeric. Numbers just below or just above the arrows indicate appropriate Kb lengths in each contig. Gene lengths and distances between genes are rounded to the nearest 6.5 kb. The site of the gap is marked with an asterisk.

Figure 2 shows a contiguous genomic sequence around chromosome 19q13.3- q13.4. Genes are represented by horizontal arrows denoting the direction of the coding sequence. Distances between genes are in base pairs.

Figure 3 shows tissue expression of the prostate/KLK-L1 gene as determined by RT-PCR. Actin and PSA are control genes. Interpretations are presented in Table 9.

Figure 4 shows the sequence of PCR product obtained with cDNA from female breast tissue using prostate/KLK-L1 primers. Primer sequences are underlined. The sequence is identical to the sequence obtained from prostatic tissue.

Figure 5 is a blot showing the results of experiments for hormonal regulation of the prostate/KLK-

L1 gene in the BT-474 breast carcinoma cell lines. DHT = dihydrotestosterone. Steroids were added at  $10^{-8}$  M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens) and PSA (up-regulated by androgens and progestins), are control genes. Prostase/KLK-L1 is up-regulated by androgens and progestins.

Figure 6 is a schematic diagram showing comparison of the genomic structure of PSA, KLK1, KLK2, zyme, neuropsin and prostase/KLK-L1 genes. Exons are shown by open boxes and introns by the connecting lines. Arrow head shows the start codons and the vertical arrow represents stop codons. Letters above boxes indicate relative positions of the catalytic triad; H denotes histidine, D aspartic acid and S serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I denotes that the intron occurs after the first nucleotide of the codon, II the intron occurs after the second nucleotide, 0 the intron occurs between codons. Numbers inside boxes indicate exon lengths in base pairs.

Figure 7 shows the genomic organization and partial genomic sequence of the KLK-L2 gene. Intronic sequences are not shown except for the splice junctions. Introns are shown with lower case letters and exons with capital letters. The start and stop codons are encircled and the exon-intron junctions are boxed. The translated amino acids of the coding region are shown underneath by a single letter abbreviation. The catalytic residues are inside triangles. Putative polyadenylation signal is underlined.

Figure 8 shows an approximate 300 Kb region of almost contiguous genomic sequence around chromosome 19q13.3- q13.4. Genes are represented by horizontal arrows denoting the direction of the coding sequence. Distances between genes are mentioned in base pairs.

Figure 9 shows the alignment of the deduced amino acid sequence of KLK-L2 with members of the kallikrein multi-gene family. Genes are (from top to bottom) : Prostase/KLK-L1, enamel matrix serine proteinase 1 (EMSP1) (GenBank accession # NP\_004908), KLK-L2, zyme (GenBank accession # Q92876), neuropsin (GenBank accession # BAA28673), trypsin-like serine protease (TLSP) (GenBank accession # BAA33404), PSA (GenBank accession # P07288), KLK2 (GenBank accession # P20151), KLK1 (GenBank accession # NP\_002248), and trypsinogen (GenBank accession # P07477). (See SEQ.ID. NOs. 68-77) Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are represented by (✱) and the 29 invariant serine protease residues by (I or ✱). Conserved areas around the catalytic triad are boxed. The predicted cleavage sites are indicated by (✂). The dotted area represents the kallikrein loop sequence. The trypsin like cleavage pattern is indicated by (✂).

Figure 10(A) shows a dendrogram of the predicted phylogenetic tree for some kallikrein genes. Neighbor-joining/UPGMA method was used to align KLK-L2 with other members of the kallikrein gene family. Gene names and accession numbers are listed in Figure 9. The tree grouped the classical kallikreins (KLK1, KLK2, and PSA) together and aligned the KLK-L2 gene in one group with EMSP, prostase, and TLSP. (B) Plot of hydrophobicity and hydrophilicity of KLK-L2.

Figure 11 is a blot showing tissue expression of KLK-L2 gene as determined by RT-PCR. Actin and PSA are control genes. Interpretations are presented in Table 12.

Figure 12 is a blot showing hormonal regulation of the KLK-L2 gene in BT-474 breast carcinoma

cell lines. DHT = dihydrotestosterone. Steroids were at  $10^{-8}$  M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens) and PSA (upregulated by androgens and progestins), are control genes. KLK-L2 is upregulated by estrogens and progestins.

Figure 13 are blots of EtBr-stained agarose gels. Total RNA was extracted from normal, benign, and cancer tissues and used to generate cDNA. PCR was performed on cDNA

Figure 14 shows an approximate 300 Kb region of almost contiguous genomic sequence around chromosome 19q13.3- q13.4. Genes are represented by horizontal arrows denoting the direction of the coding sequence. Gene lengths and distances between genes are rounded to the nearest 0.5 kb. The site of the gap is marked with an asterisk. Telomeric to TLSP there are likely another three kallikrein-like genes.

Figure 15 shows the genomic organization and partial genomic sequence of the KLK-L3 gene. Intronic sequences are not shown except for the splice junctions. Introns are shown with lower case letters and exons with capital letters. For the full sequence, see SEQ.ID. NO. 21. The start and stop codons are encircled and the exon-intron junctions are boxed. The translated amino acids of the coding region are shown underneath by a single letter abbreviation. The catalytic residues are inside triangles. Putative polyadenylation signal is underlined.

Figure 16 is a plot of hydrophobicity and hydrophilicity, comparing the pattern of the KLK-L3 with that of the zyme gene. Note the hydrophobic region around the first twenty amino acids, likely representing the signal peptide.

Figure 17 is an alignment of the deduced amino acid sequence of KLK-L3 with members of the kallikrein multi-gene family. Genes are (from top to bottom and in brackets is the GenBank accession #): PSA (P07288), KLK2 (P20151), KLK1 (NP002248), trypsinogen (P07477), KLK-L3 (AF135026), trypsin-like serine protease (TLSP) (BAA33404), neuropsin (BAA28673), zyme (Q92876), human stratum corneum chymotryptic enzyme (HSCCE) (AAD49718), and/prostate/CLK-L1 (AAD21581). (See SEQ.ID. NOs. 78 to 84). Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are bold and in italics, and the 29 invariant serine protease residues are denoted by ( $\diamond$ ). Cysteine residues are marked by ( $\bullet$ ). Conserved areas around the catalytic triad are highlighted in black. The arrow heads ( $\blacktriangleright$ ) represent the potential cleavage sites. The dotted area represents the kallikrein loop sequence.

Figure 18 is a dendrogram of the predicted phylogenetic tree for some serine proteases and kallikrein genes. Neighbor-joining/UPGMA method was used to align KLK-L3 with other members of the kallikrein gene family. Gene names and accession numbers are listed in Figure 17. The tree grouped the classical kallikreins (KLK1, KLK2, and PSA) together and aligned the KLK-L3 gene in one group with TLSP, neuropsin, and NES 1 genes. KLK-L4 (SEQ.ID.NO. 43) lies further telomeric to TLSP (21).

Figure 19 is a blot showing tissue expression of the KLK-L3 gene as determined by RT-PCR. Actin and PSA are control genes.

Figure 20 shows hormonal regulation of the KLK-L3 gene in the BT-474 breast carcinoma cell line. DHT = dihydrotestosterone. Steroids were at  $10^{-8}$  M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens) and PSA (upregulated by androgens and progestins),

are control genes. KLK-L3 is upregulated by progestins, estrogens and androgens, in that order.

Figure 21 is a schematic diagram showing the comparison of the genomic structure of PSA, KLK2, neuropsin, NES1, and KLK-L3 genes. Exons are shown by black boxes and introns by the connecting lines. Arrowheads show the start codon, and arrows show the stop codon. Letters above boxes indicate relative positions of the catalytic triad; H denotes histidine, D aspartic acid and S serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I denotes that the intron occurs after the first nucleotide of the codon, II the intron occurs after the second nucleotide, 0 the intron occurs between codons. Numbers inside boxes indicate exon lengths in base pairs.

Figure 22 shows a comparative genomic structure of the ESTs (Table 16), the clone from The German Genome Project, and the long form of KLK-L4. Exons are represented by solid bars and introns by the connecting lines. Exon numbers on top of solid bars refer to GenBank submission #AF135024. The EST IDs represent GenBank accession numbers. Asterisks represent the positions of stop codons. Horizontal arrows indicate the direction of the PCR primers (described in Table 15) and arrowheads their position along the exons. Vertical dotted lines show alignment of identical fragments.

Figure 23 shows tissue expression of the KLK-L4 gene as determined by RT-PCR. Actin and PSA are control genes. KLK-L4 is highly expressed in breast, prostate, salivary gland and testis.

Figure 24 in the Upper Panel is a Diagram showing the comparative genomic structure of the long KLK-L4 form and the short KLK-L4 variant. Exons are represented by boxes and introns by the connecting lines. Exon numbers refer to SEQ. ID. NO. 43 and GenBank Accession No. AF135024. The black region indicates the extra fragment (214 bp) that is found in the long, but not in the short form of the gene. The positions of the stop codons of the two forms are marked with asterisks. Frame shifting occurs as a result of utilization of an alternative splice site, and a stop codon is generated at the beginning of exon 4 in the short form. The Lower Panel shows PCR products of the amplification of the KLK-L4 gene using L4-R1 and L4-X1 primers (Figure 22 and Table 15). Note the predominant long form and a minor band representing the short form of KLK-L4 mRNA. (M); Markers with sizes in bp shown on the left. Tissues used: (1), salivary gland; (2), mammary gland; (3), prostate; (4), testis; (5), uterus; (6), breast cancer tissue; (7), negative control.

Figure 25 shows the genomic organization and partial genomic sequence of the KLK-L4 gene. Intronic sequences are not shown except for the splice junction areas. Introns are shown with lower case letters and exons with capital letters. For full sequence, see SEQ. ID. NO.43 or GenBank Accession #AF135024. The start and stop codons are encircled and the exon-intron junctions are underlined. The translated amino acids of the coding region are shown underneath by a single letter abbreviation. The catalytic residues are boxed. The putative polyadenylation signal is underlined.

Figure 26 is a plot of hydrophobicity and hydrophilicity of the KLK-L4 protein, as compared with the glandular kallikrein gene 2 (KLK2). Note the hydrophobic region at the amino terminus, suggesting presence of a signal peptide.

Figure 27 shows an alignment of the deduced amino acid sequence of KLK-L4 with members of the kallikrein multi-gene family. Genes are (from top to bottom, and in brackets are the GenBank accession



#): KLK-L1/protease (AAD21581), enamel matrix serine proteinase 1 (EMSP) (NP\_004908), KLK-L2 (AF135028), PSA (P07288), KLK2 (P20151), KLK1 (NP\_002248), trypsinogen (P07477), zyme (Q92876), KLK-L4 (AF135024), trypsin-like serine protease (TLSP) (BAA33404), KLK-L3 (AF135026), neuropsin (BAA28673), and the normal epithelial cell-specific 1 gene (NES1) (O43240). (See SEQ.ID. NOs. 78-88). Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are typed in bold, and conserved motifs around them are highlighted in grey. The 29 invariant serine protease residues are denoted by (●), and the cysteine residues by (◆). The predicted cleavage sites are indicated by (▲). The dotted area represents the kallikrein loop sequence. The trypsin-like cleavage pattern of KLK-L4 with the D residue, is indicated by (⊙).

Figure 28 shows an approximate 300 Kb region of almost contiguous genomic sequence around chromosome 19q13.3- q13.4. Genes are represented by horizontal arrows denoting the direction of the coding sequence. Their lengths are shown on top of each arrow. Distances between genes are mentioned in base pairs below the arrows. The distance between KLK1 and PSA is not accurately known. For gene names, see under abbreviations.

Figure 29 shows a dendrogram of the predicted phylogenetic tree for some kallikrein and serine protease genes. The neighbor-joining/UPGMA method was used to align KLK-L4 with other serine proteases and members of the kallikrein gene family. The tree grouped the classical kallikreins (KLK1, KLK2, and PSA) together and aligned the KLK-L4 gene in one group with zyme, NES1, neuropsin, KLK-L3, and TLSP. Other serine proteases were aligned in different groups, as shown.

Figure 30 is a blot showing the hormonal regulation of the KLK-L4 gene in the BT-474 breast carcinoma cell line. DIHT = dihydrotestosterone. Steroids were added at  $10^{-8}$  M final concentrations. Actin (not regulated by steroid hormones), pS2 (up-regulated by estrogens) and PSA (upregulated by androgens and progestins) are control genes. KLK-L4 is up-regulated by androgens and progestins and to a lesser extent by estrogens.  $H_2O$  was used to check for PCR specificity in all PCR reactions. For more details, see text.

Figure 31 is a schematic diagram showing the comparison of the genomic structure of PSA, KLK2, neuropsin, NES1, and KLK-L4 genes. Exons are shown by black boxes and introns by the connecting lines. The arrowhead shows the start codons and the arrow the stop codons. Letters above boxes indicate the relative positions of the amino acids of the catalytic triad; H denotes histidine, D aspartic acid and S serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I, the intron occurs after the first nucleotide of the codon, II the intron occurs after the second nucleotide, 0 the intron occurs between codons. Numbers inside boxes indicate exon lengths in base pairs. The question mark indicates the possibility of more untranslated bases.

Figure 32 is a diagram showing the comparative genomic structure of the three splice forms of KLK-L5; the classical kallikrein form, related protein-1, and related protein-2. Exons are represented by solid bars and introns by the connecting lines. Exon numbers refer to SEQ.ID. NO.56 and GenBank Accession #AF135025. Start codons are represented by the inverted arrowhead (▼) and stop codons are represented by asterisks (\*). Primer locations are represented by vertical arrowheads (▲) and their

directions by horizontal arrows. For primer sequences and codes see Table 17 and SEQ.ID. NOS. 61-64, and 9-12.

Figure 33 shows the genomic organization and partial genomic sequence of the KLK-L5 gene. Intronic sequences are not shown except for short sequences around the splice junctions. Introns are shown with lower case letters and exons with capital letters. For full sequence, see SEQ.ID.NO. 56. The start and stop codons are encircled and the exon-intron junctions are underlined. The translated amino acids of the coding region are shown underneath by a single letter abbreviation. The catalytic residues are boxed. Putative polyadenylation signal is underlined. The extra intron of the related protein-1 form is represented by non-bold capital letters between brackets. When this intron is spliced, the frame continues with codon AAC (asparagine, N, instead of lysine, K) until it encounters the stop codon TAA (encircled).

Figure 34 is a schematic diagram showing the comparison of the genomic structure of PSA, KLK2, neuropsin, NES1, KLK-L4 and KLK-L5 genes. Exons are shown by solid bars and introns by the connecting lines. Arrowhead marks the site of the start codon, and the arrow represents the stop codon. Letters above boxes indicate relative positions of the catalytic triad; H denotes histidine, D aspartic acid and S serine. Roman numbers indicate intron phases. The intron phase refers to the location of the intron within the codon; I denotes that the intron occurs after the first nucleotide of the codon, II the intron occurs after the second nucleotide, 0 the intron occurs between codons. Numbers inside boxes indicate exon lengths in base pairs. Question marks indicate that exact length is not accurately known.

Figure 35 is a plot of hydrophobicity and hydrophilicity of KLK-L5 protein compared to prostate specific antigen (PSA). The hydrophobic N-terminus may harbor a signal and activation peptide.

Figure 36 shows an alignment of the deduced amino acid sequence of KLK-L5 with members of the kallikrein multigene family. (See SEQ.ID. NOS. 78-81, 83, 84). Dashes represent gaps to bring the sequences to better alignment. The residues of the catalytic triad are represented by bold letters, and the 29 invariant serine protease residues are marked with (•). The cysteine residues are marked by (♦). Conserved areas are highlighted in grey. The predicted cleavage sites in signal peptide are indicated by (♣). The dotted area represents the kallikrein loop sequence. A vertical arrow marks the trypsin like cleavage site.

Figure 37 is a dendrogram of the predicted phylogenetic tree for some serine proteases and other kallikrein proteins. Neighbor-joining/UPGMA method was used to align KLK-L5 with other serine proteases and members of the kallikrein gene family. The tree grouped the classical kallikreins (hK1, hK2, and PSA) together and aligned the KLK-L5 protein in one group with NES1 and neuropsin. Other serine proteases were aligned in different groups.

Figure 38 shows tissue expression of the KLK-L5 gene as determined by RT-PCR. The upper band (905 base pairs, bp) is the classical form (see Figure 32, the middle (776 bp) the related protein-1, and the lower band (644 bp) the related protein-2. For splice variant discussion see text. The primers used were L5-F2 and L5-R2, as shown in Table 17.

Figure 39 shows hormonal regulation of the KLK-L5 gene in the LnCaP prostatic carcinoma cell line, BT-474 and T-47D breast carcinoma cell lines. Steroids were at  $10^{-8}$  M final concentration. Actin (not

regulated by steroid hormones) was used as a control gene. Note detection of three isoforms only in LNCaP.

Figure 40 shows the expression of the KLK-L5 gene in breast cancer (1-17) and normal (18) tissues. Note complete absence of expression in 12 cancer tissues. For isoforms see also Figure 38.

5 Figure 41 shows the full structure of a KLK-L6 nucleic acid molecule;

Figure 42 is a plot of hydrophobicity and hydrophilicity of KLK-L6 protein compared to prostate specific antigen (PSA).

10 Figure 43 shows an alignment of the deduced amino acid sequence of KLK-L6 with members of the kallikrein multigene family. (See SEQ.ID. NOs. 78-81, 83, 84). Dashes represent gaps to bring the sequences to better alignment.

Figure 44 is a dendrogram of the predicted phylogenetic tree for some serine proteases and other kallikrein proteins. Neighbor-joining/UPGMA method was used to align KLK-L6 with other serine proteases and members of the kallikrein gene family.

#### DETAILED DESCRIPTION OF THE INVENTION

15 In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See for example, Sambrook, Fritsch, & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization B.D. Hames & S.J. Higgins eds. (1985); Transcription and Translation B.D. Hames & S.J. Higgins eds (1984); Animal Cell Culture R.I. Freshney, ed. (1986); Immobilized Cells and enzymes IRL Press, (1986); and B. Perbal, A Practical Guide to Molecular Cloning (1984).

##### 1. Nucleic Acid Molecules of the Invention

25 As hereinbefore mentioned, the invention provides an isolated nucleic acid molecule having a sequence encoding a KLK-L Protein. The term "isolated" refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical reactants, or other chemicals when chemically synthesized. An "isolated" nucleic acid may also be free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid molecule) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded. In an embodiment, a nucleic acid molecule encodes a KLK-L Protein comprising an amino acid sequence as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, preferably a nucleic acid molecule comprising a nucleic acid sequence as shown in SEQ.ID.NO. 1, 13, 21, 43, 56, or 65.

35 The invention includes nucleic acid sequences complementary to a nucleic acid encoding a KLK-L Protein comprising an amino acid sequence as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, preferably the nucleic acid sequences complementary to a full nucleic acid sequence shown in SEQ.ID.NO. 1, 13, 21, 43, 56, or 65.

The invention includes nucleic acid molecules having substantial sequence identity or homology to nucleic acid sequences of the invention or encoding proteins having substantial identity or similarity to the amino acid sequence shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67. Preferably, the nucleic acids have substantial sequence identity for example at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% nucleic acid identity; more preferably 90% nucleic acid identity; and most preferably at least 95%, 96%, 97%, 98%, or 99% sequence identity. "Identity" as known in the art and used herein, is a relationship between two or more amino acid sequences or two or more nucleic acid sequences, as determined by comparing the sequences. It also refers to the degree of sequence relatedness between amino acid or nucleic acid sequences, as the case may be, as determined by the match between strings of such sequences. Identity and similarity are well known terms to skilled artisans and they can be calculated by conventional methods (for example see Computational Molecular Biology, Lesk, A.M. ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W. ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M. and Griffin, H.G. eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G. Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J. eds. M. Stockton Press, New York, 1991, Carillo, H. and Lipman, D., SIAM J. Applied Math. 48:1073, 1988). Methods which are designed to give the largest match between the sequences are generally preferred. Methods to determine identity and similarity are codified in publicly available computer programs including the GCG program package (Devereux J. et al., Nucleic Acids Research 12(1): 387, 1984); BLASTP, BLASTN, and FASTA (Altschul, S.F. et al. J. Molec. Biol. 215: 403-410, 1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S. et al. NCBI NLM NIH Bethesda, Md. 20894; Altschul, S. et al. J. Mol. Biol. 215: 403-410, 1990).

Isolated nucleic acid molecules encoding a KLK-L Protein, and having a sequence which differs from a nucleic acid sequence of the invention due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent proteins (e.g., a KLK-L Protein) but differ in sequence from the sequence of a KLK-L Protein due to degeneracy in the genetic code. As one example, DNA sequence polymorphisms within the nucleotide sequence of a KLK-L Protein may result in silent mutations which do not affect the amino acid sequence. Variations in one or more nucleotides may exist among individuals within a population due to natural allelic variation. Any and all such nucleic acid variations are within the scope of the invention. DNA sequence polymorphisms may also occur which lead to changes in the amino acid sequence of a KLK-L Protein. These amino acid polymorphisms are also within the scope of the present invention.

Another aspect of the invention provides a nucleic acid molecule which hybridizes under stringent conditions, preferably high stringency conditions to a nucleic acid molecule which comprises a sequence which encodes a KLK-L Protein having an amino acid sequence shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C,

followed by a wash of  $2.0 \times \text{SSC}$  at  $50^\circ\text{C}$  may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about  $0.2 \times \text{SSC}$  at  $50^\circ\text{C}$ . In addition, the temperature in the wash step can be at high stringency conditions, at about  $65^\circ\text{C}$ .

It will be appreciated that the invention includes nucleic acid molecules encoding a KLK-L Related Protein including truncations of a KLK-L Protein, and analogs of a KLK-L Protein as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention. (See for example, splice variants of KLK-L5, SEQ.ID.NO. 58, 59, and 60.)

An isolated nucleic acid molecule of the invention which comprises DNA can be isolated by preparing a labelled nucleic acid probe based on all or part of a nucleic acid sequence of the invention. The labeled nucleic acid probe is used to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). For example, a cDNA library can be used to isolate a cDNA encoding a KLK-L Related Protein by screening the library with the labeled probe using standard techniques. Alternatively, a genomic DNA library can be similarly screened to isolate a genomic clone encompassing a gene encoding a KLK-L Related Protein. Nucleic acids isolated by screening of a cDNA or genomic DNA library can be sequenced by standard techniques.

An isolated nucleic acid molecule of the invention which is DNA can also be isolated by selectively amplifying a nucleic acid encoding a KLK-L Related Protein using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleotide sequence of the invention for use in PCR. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

An isolated nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding a KLK-L Related Protein into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a KLK-L Related Protein. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can be transcribed *in vitro* with T7 polymerase, and the resultant RNA can be isolated by conventional techniques.

Nucleic acid molecules of the invention may be chemically synthesized using standard techniques. Methods of chemically synthesizing polydeoxynucleotides are known, including but not limited to solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

Determination of whether a particular nucleic acid molecule encodes a KLK-L Related Protein can be accomplished by expressing the cDNA in an appropriate host cell by standard techniques, and testing the expressed protein in the methods described herein. A cDNA encoding a KLK-L Related Protein can be sequenced by standard techniques, such as dideoxynucleotide chain termination or Maxam-Gilbert  
5 chemical sequencing, to determine the nucleic acid sequence and the predicted amino acid sequence of the encoded protein.

The initiation codon and untranslated sequences of a KLK-L Related Protein may be determined using computer software designed for the purpose, such as PC/Gene (IntelliGenetics Inc., Calif.). The intron-exon structure and the transcription regulatory sequences of a gene encoding a KLK-L Related  
10 Protein may be confirmed by using a nucleic acid molecule of the invention encoding a KLK-L Related Protein to probe a genomic DNA clone library. Regulatory elements can be identified using standard techniques. The function of the elements can be confirmed by using these elements to express a reporter gene such as the lacZ gene which is operatively linked to the elements. These constructs may be introduced into cultured cells using conventional procedures or into non-human transgenic animal models. In addition  
15 to identifying regulatory elements in DNA, such constructs may also be used to identify nuclear proteins interacting with the elements, using techniques known in the art.

In a particular embodiment of the invention, the nucleic acid molecules isolated using the methods described herein are mutant *klk-l* gene alleles. The mutant alleles may be isolated from individuals either known or proposed to have a genotype which contributes to the symptoms of for example, cancer (e.g.,  
20 breast, testicular, brain, colon, and prostate cancer). Mutant alleles and mutant allele products may be used in therapeutic and diagnostic methods described herein. For example, a cDNA of a mutant *klk-l* gene may be isolated using PCR as described herein, and the DNA sequence of the mutant allele may be compared to the normal allele to ascertain the mutation(s) responsible for the loss or alteration of function of the mutant gene product. A genomic library can also be constructed using DNA from an individual suspected  
25 of or known to carry a mutant allele, or a cDNA library can be constructed using RNA from tissue known, or suspected to express the mutant allele. A nucleic acid encoding a normal *klk-l* gene or any suitable fragment thereof, may then be labeled and used as a probe to identify the corresponding mutant allele in such libraries. Clones containing mutant sequences can be purified and subjected to sequence analysis. In addition, an expression library can be constructed using cDNA from RNA isolated from a tissue of an  
30 individual known or suspected to express a mutant *klk-l* allele. Gene products made by the putatively mutant tissue may be expressed and screened, for example using antibodies specific for a KLK-L Related Protein as described herein. Library clones identified using the antibodies can be purified and subjected to sequence analysis.

The sequence of a nucleic acid molecule of the invention, or a fragment of the molecule, may be  
35 inverted relative to its normal presentation for transcription to produce an antisense nucleic acid molecule. An antisense nucleic acid molecule may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art.

## 2. Proteins of the Invention

An amino acid sequence of a KLK-L Protein comprises a sequence as shown in Tables 1 to 5 or SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67.

In addition to proteins comprising an amino acid sequence as shown in Tables 1 to 5 or SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, the proteins of the present invention include truncations of a KLK-L Protein, analogs of a KLK-L Protein, and proteins having sequence identity or similarity to a KLK-L Protein, and truncations thereof as described herein (i.e. included in KLK-L Related Proteins). Truncated proteins may comprise peptides of between 3 and 70 amino acid residues, ranging in size from a tripeptide to a 70 mer polypeptide.

The truncated proteins may have an amino group (-NH<sub>2</sub>), a hydrophobic group (for example, carbobenzoxy, dansyl, or T-butyloxycarbonyl), an acetyl group, a 9-fluorenylmethoxy-carbonyl (PMOC) group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the amino terminal end. The truncated proteins may have a carboxyl group, an amido group, a T-butyloxycarbonyl group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the carboxy terminal end.

The proteins of the invention may also include analogs of a KLK-L Protein, and/or truncations thereof as described herein, which may include, but are not limited to a KLK-L Protein, containing one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of a KLK-L Protein amino acid sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog is preferably functionally equivalent to a KLK-L Protein. Non-conserved substitutions involve replacing one or more amino acids of the KLK-L Protein amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into a KLK-L Protein. Amino acid insertions may consist of single amino acid residues or sequential amino acids ranging from 2 to 15 amino acids in length.

Deletions may consist of the removal of one or more amino acids, or discrete portions from a KLK-L Protein sequence. The deleted amino acids may or may not be contiguous. The lower limit length of the resulting analog with a deletion mutation is about 10 amino acids, preferably 20 to 40 amino acids.

The proteins of the invention include proteins with sequence identity or similarity to a KLK-L Protein and/or truncations thereof as described herein. Such KLK-L Proteins include proteins whose amino acid sequences are comprised of the amino acid sequences of KLK-L Protein regions from other species that hybridize under selected hybridization conditions (see discussion of stringent hybridization conditions herein) with a probe used to obtain a KLK-L Protein. These proteins will generally have the same regions which are characteristic of a KLK-L Protein. Preferably a protein will have substantial sequence identity for example, about 30%, 35%, 40%, 45%, 50%, 60%, 65%, 70%, 75%, 80%, or 85% identity, preferably 90% identity, more preferably at least 95%, 96%, 97%, 98%, or 99% identity, and most preferably 98% identity with an amino acid sequence shown in Tables 1 to 5 or SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57,

58, 59, 60, 66, or 67.

A percent amino acid sequence homology, similarity or identity is calculated as the percentage of aligned amino acids that match the reference sequence using known methods as described herein.

5 The invention also contemplates isoforms of the proteins of the invention. An isoform contains the same number and kinds of amino acids as a protein of the invention, but the isoform has a different molecular structure. Isoforms contemplated by the present invention preferably have the same properties as a protein of the invention as described herein.

The present invention also includes KLK-L Related Proteins conjugated with a selected protein, or a marker protein (see below) to produce fusion proteins. Additionally, immunogenic portions of a KLK-L Protein and a KLK-L Protein Related Protein are within the scope of the invention.

10 A KLK-L Related Protein of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention having a sequence which encodes a KLK-L Related Protein of the invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence. Suitable regulatory sequences may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. The necessary regulatory sequences may be supplied by the native KLK-L Protein and/or its flanking regions.

25 The invention further provides a recombinant expression vector comprising a DNA nucleic acid molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is linked to a regulatory sequence in a manner which allows for expression, by transcription of the DNA molecule, of an RNA molecule which is antisense to the nucleic acid sequence of a protein of the invention or a fragment thereof. Regulatory sequences linked to the antisense nucleic acid can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance a viral promoter and/or enhancer, or regulatory sequences can be chosen which direct tissue or cell type specific expression of antisense RNA.

35 The recombinant expression vectors of the invention may also contain a marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs,  $\beta$ -galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. The markers can be introduced on a separate vector from the nucleic acid of interest.



The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

The recombinant expression vectors may be introduced into host cells to produce a transformant host cell. "Transformant host cells" include host cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" encompass the introduction of a nucleic acid (e.g. a vector) into a cell by one of many standard techniques. Prokaryotic cells can be transformed with a nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. A nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells, or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

A host cell may also be chosen which modulates the expression of an inserted nucleic acid sequence, or modifies (e.g. glycosylation or phosphorylation) and processes (e.g. cleaves) the protein in a desired fashion. Host systems or cell lines may be selected which have specific and characteristic mechanisms for post-translational processing and modification of proteins. For example, eukaryotic host cells including CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and WI38 may be used. For long-term high-yield stable expression of the protein, cell lines and host systems which stably express the gene product may be engineered.

Host cells and in particular cell lines produced using the methods described herein may be particularly useful in screening and evaluating compounds that modulate the activity of a KLK-L Related Protein.

The proteins of the invention may also be expressed in non-human transgenic animals including but not limited to mice, rats, rabbits, guinea pigs, micro-pigs, goats, sheep, pigs, non-human primates (e.g. baboons, monkeys, and chimpanzees) [see Hammer et al. (Nature 315:680-683, 1985), Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:44384442, 1985), Palmiter and Brinster (Cell. 41:343-345, 1985) and U.S. Patent No. 4,736,866)]. Procedures known in the art may be

used to introduce a nucleic acid molecule of the invention encoding a KLK-L Related Protein into animals to produce the founder lines of transgenic animals. Such procedures include pronuclear microinjection, retrovirus mediated gene transfer into germ lines, gene targeting in embryonic stem cells, electroporation of embryos, and sperm-mediated gene transfer.

5 The present invention contemplates a transgenic animal that carries the *KLK-L* gene in all their cells, and animals which carry the transgene in some but not all their cells. The transgene may be integrated as a single transgene or in concatamers. The transgene may be selectively introduced into and activated in specific cell types (See for example, Lasko et al, 1992 Proc. Natl. Acad. Sci. USA 89: 6236). The transgene may be integrated into the chromosomal site of the endogenous gene by gene targeting. The transgene may be selectively introduced into a particular cell type inactivating the endogenous gene in that cell type (See 10 Gu et al Science 265: 103-106).

The expression of a recombinant KLK-L Related Protein in a transgenic animal may be assayed using standard techniques. Initial screening may be conducted by Southern Blot analysis, or PCR methods to analyze whether the transgene has been integrated. The level of mRNA expression in the tissues of 15 transgenic animals may also be assessed using techniques including Northern blot analysis of tissue samples, *in situ* hybridization, and RT-PCR. Tissue may also be evaluated immunocytochemically using antibodies against KLK-L Protein.

Proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

N-terminal or C-terminal fusion proteins comprising a KLK-L Related Protein of the invention conjugated with other molecules, such as proteins, may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of a KLK-L Related Protein, and the sequence of a selected 25 protein or marker protein with a desired biological function. The resultant fusion proteins contain KLK-L Protein fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include immunoglobulins, glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

### 3. Antibodies

30 KLK-L Related Proteins of the invention can be used to prepare antibodies specific for the proteins. Antibodies can be prepared which bind a distinct epitope in an unconserved region of the protein. An unconserved region of the protein is one that does not have substantial sequence homology to other proteins. A region from a conserved region such as a well-characterized domain can also be used to prepare an antibody to a conserved region of a KLK-L Related Protein. Antibodies having specificity for a KLK-L 35 Related Protein may also be raised from fusion proteins created by expressing fusion proteins in bacteria as described herein.

The invention can employ intact monoclonal or polyclonal antibodies, and immunologically active fragments (e.g. a Fab, (Fab)<sub>2</sub> fragment, or Fab expression library fragments and epitope-binding fragments

thereof), an antibody heavy chain, and antibody light chain, a genetically engineered single chain Fv molecule (Ladner et al, U.S. Pat. No. 4,946,778), or a chimeric antibody, for example, an antibody which contains the binding specificity of a murine antibody, but in which the remaining portions are of human origin. Antibodies including monoclonal and polyclonal antibodies, fragments and chimeras, may be prepared using methods known to those skilled in the art.

#### 4. Applications of the Nucleic Acid Molecules, KLK-L Related Proteins, and Antibodies of the Invention

The nucleic acid molecules, KLK-L Related Proteins, and antibodies of the invention may be used in the prognostic and diagnostic evaluation of cancer (e.g. breast, testicular, and prostate cancer) or other conditions, and the identification of subjects with a predisposition to cancer (Section 4.1.1 and 4.1.2). Methods for detecting nucleic acid molecules and KLK-L Related Proteins of the invention, can be used to monitor conditions including cancer, by detecting KLK-L Related Proteins and nucleic acid molecules encoding KLK-L Related Proteins. It would also be apparent to one skilled in the art that the methods described herein may be used to study the developmental expression of KLK-L Related Proteins and, accordingly, will provide further insight into the role of KLK-L Related Proteins. The applications of the present invention also include methods for the identification of compounds that modulate the biological activity of *KLK-L* or KLK-L Related Proteins (Section 4.2). The compounds, antibodies etc. may be used for the treatment of cancer (Section 4.3).

#### 4.1 Diagnostic Methods

A variety of methods can be employed for the diagnostic and prognostic evaluation of conditions including cancer (e.g. breast, testicular, and prostate cancer), and the identification of subjects with a predisposition to such conditions. Such methods may, for example, utilize nucleic acid molecules of the invention, and fragments thereof, and antibodies directed against KLK-L Related Proteins, including peptide fragments. In particular, the nucleic acids and antibodies may be used, for example, for: (1) the detection of the presence of *KLK-L* mutations, or the detection of either over- or under-expression of *KLK-L* mRNA relative to a non-disorder state or the qualitative or quantitative detection of alternatively spliced forms of *KLK-L* transcripts which may correlate with certain conditions or susceptibility toward such conditions; and (2) the detection of either an over- or an under-abundance of KLK-L Related Proteins relative to a non-disorder state or the presence of a modified (e.g., less than full length) KLK-L Protein which correlates with a disorder state, or a progression toward a disorder state.

The methods described herein may be performed by utilizing pre-packaged diagnostic kits comprising at least one specific *KLK-L* nucleic acid or antibody described herein, which may be conveniently used, e.g., in clinical settings, to screen and diagnose patients and to screen and identify those individuals exhibiting a predisposition to developing a disorder.

Nucleic acid-based detection techniques are described, below, in Section 4.1.1. Peptide detection techniques are described, below, in Section 4.1.2. The samples that may be analyzed using the methods of the invention include those which are known or suspected to express *KLK-L* or contain KLK-L Related Proteins. The samples may be derived from a patient or a cell culture, and include but are not limited to

biological fluids, tissue extracts, freshly harvested cells, and lysates of cells which have been incubated in cell cultures.

Oligonucleotides or longer fragments derived from any of the nucleic acid molecules of the invention may be used as targets in a microarray. The microarray can be used to simultaneously monitor the expression levels of large numbers of genes and to identify genetic variants, mutations, and polymorphisms. The information from the microarray may be used to determine gene function, to understand the genetic basis of a disorder, to diagnose a disorder, and to develop and monitor the activities of therapeutic agents.

The preparation, use, and analysis of microarrays are well known to a person skilled in the art. (See, for example, Brennan, T. M. et al. (1995) U.S. Pat. No. 5,474,796; Schena, et al. (1996) Proc. Natl. Acad. Sci. 93:10614-10619; Baldeschweiler et al. (1995), PCT Application WO95/251116; Shalon, D. et al. (1995) PCT application WO95/35505; Heller, R. A. et al. (1997) Proc. Natl. Acad. Sci. 94:2150-2155; and Heller, M. J. et al. (1997) U.S. Pat. No. 5,605,662.)

#### 4.1.1 Methods for Detecting Nucleic Acid Molecules of the Invention

The nucleic acid molecules of the invention allow those skilled in the art to construct nucleotide probes for use in the detection of nucleic acid sequences of the invention in samples. Suitable probes include nucleic acid molecules based on nucleic acid sequences encoding at least 5 sequential amino acids from regions of a KLK-L Protein, preferably they comprise 15 to 30 nucleotides. A nucleotide probe may be labeled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as  $^{32}\text{P}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$  or the like. Other detectable substances which may be used include antigens that are recognized by a specific labeled antibody, fluorescent compounds, enzymes, antibodies specific for a labeled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labeled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleic acid probes may be used to detect genes, preferably in human cells, that encode KLK-L Related Proteins. The nucleotide probes may also be useful in the diagnosis of cancer; in monitoring the progression of cancer; or monitoring a therapeutic treatment.

The probe may be used in hybridization techniques to detect genes that encode KLK-L Related Proteins. The technique generally involves contacting and incubating nucleic acids (e.g. recombinant DNA molecules, cloned genes) obtained from a sample from a patient or other cellular source with a probe of the present invention under conditions favorable for the specific annealing of the probes to complementary sequences in the nucleic acids. After incubation, the non-annealed nucleic acids are removed, and the presence of nucleic acids that have hybridized to the probe if any are detected.

The detection of nucleic acid molecules of the invention may involve the amplification of specific gene sequences using an amplification method such as PCR, followed by the analysis of the amplified molecules using techniques known to those skilled in the art. Suitable primers can be routinely designed

by one of skill in the art.

Genomic DNA may be used in hybridization or amplification assays of biological samples to detect abnormalities involving *klk-l* structure, including point mutations, insertions, deletions, and chromosomal rearrangements. For example, direct sequencing, single stranded conformational polymorphism analyses, heteroduplex analysis, denaturing gradient gel electrophoresis, chemical mismatch cleavage, and oligonucleotide hybridization may be utilized.

Genotyping techniques known to one skilled in the art can be used to type polymorphisms that are in close proximity to the mutations in a *klk-l* gene. The polymorphisms may be used to identify individuals in families that are likely to carry mutations. If a polymorphism exhibits linkage disequilibrium with mutations in a *klk-l* gene, it can also be used to screen for individuals in the general population likely to carry mutations. Polymorphisms which may be used include restriction fragment length polymorphisms (RFLPs), single-base polymorphisms, and simple sequence repeat polymorphisms (SSLPs).

A probe of the invention may be used to directly identify RFLPs. A probe or primer of the invention can additionally be used to isolate genomic clones such as YACs, BACs, PACs, cosmids, phage or plasmids. The DNA in the clones can be screened for SSLPs using hybridization or sequencing procedures.

Hybridization and amplification techniques described herein may be used to assay qualitative and quantitative aspects of *klk-l* expression. For example, RNA may be isolated from a cell type or tissue known to express *klk-l* and tested utilizing the hybridization (e.g. standard Northern analyses) or PCR techniques referred to herein. The techniques may be used to detect differences in transcript size which may be due to normal or abnormal alternative splicing. The techniques may be used to detect quantitative differences between levels of full length and/or alternatively splice transcripts detected in normal individuals relative to those individuals exhibiting cancer symptoms or other disease conditions.

The primers and probes may be used in the above described methods *in situ* i.e. directly on tissue sections (fixed and/or frozen) of patient tissue obtained from biopsies or resections.

#### 4.1.2 Methods for Detecting KLK-L Related Proteins

Antibodies specifically reactive with a KLK-L Related Protein, or derivatives, such as enzyme conjugates or labeled derivatives, may be used to detect KLK-L Related Proteins in various samples (e.g. biological materials). They may be used as diagnostic or prognostic reagents and they may be used to detect abnormalities in the level of KLK-L Related Proteins expression, or abnormalities in the structure, and/or temporal, tissue, cellular, or subcellular location of a KLK-L Related Protein. Antibodies may also be used to screen potentially therapeutic compounds *in vitro* to determine their effects on cancer, and other conditions. *In vitro* immunoassays may also be used to assess or monitor the efficacy of particular therapies. The antibodies of the invention may also be used *in vitro* to determine the level of *KLK-L* expression in cells genetically engineered to produce a KLK-L Related Protein.

The antibodies may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of a KLK-L Related Protein and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation,

latex agglutination, hemagglutination, and histochemical tests. The antibodies may be used to detect and quantify KLK-L Related Proteins in a sample in order to determine its role in particular cellular events or pathological states, and to diagnose and treat such pathological states.

In particular, the antibodies of the invention may be used in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect a KLK-L Related Protein, to localize it to particular cells and tissues, and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect a KLK-L Related Protein. Generally, an antibody of the invention may be labeled with a detectable substance and a KLK-L Related Protein may be localised in tissues and cells based upon the presence of the detectable substance. Examples of detectable substances include, but are not limited to, the following: radioisotopes (e.g.,  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), luminescent labels such as luminol; enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase, acetylcholinesterase), biotinyl groups (which can be detected by marked avidin e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or calorimetric methods), predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached via spacer arms of various lengths to reduce potential steric hindrance. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

The antibody or sample may be immobilized on a carrier or solid support which is capable of immobilizing cells, antibodies etc. For example, the carrier or support may be nitrocellulose, or glass, polyacrylamides, gabbros, and magnetite. The support material may have any possible configuration including spherical (e.g. bead), cylindrical (e.g. inside surface of a test tube or well, or the external surface of a rod), or flat (e.g. sheet, test strip). Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against KLK-L Related Protein. By way of example, if the antibody having specificity against a KLK-L Related Protein is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labeled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, a KLK-L Related Protein may be localized by radioautography. The results of radioautography may be quantitated by determining the density of particles in the radioautographs by various optical methods, or by counting the grains.

#### **4.2 Methods for Identifying or Evaluating Substances/Compounds**

The methods described herein are designed to identify substances that modulate the biological activity of a KLK-L Related Protein including substances that bind to KLK-L Related Proteins, or bind to other proteins that interact with a KLK-L Related Protein, to compounds that interfere with, or enhance the interaction of a KLK-L Related Protein and substances that bind to the KLK-L Related Protein or other proteins that interact with a KLK-L Related Protein. Methods are also utilized that identify compounds that bind to *KLK-L* regulatory sequences.

The substances and compounds identified using the methods of the invention include but are not limited to peptides such as soluble peptides including Ig-tailed fusion peptides, members of random peptide libraries and combinatorial chemistry-derived molecular libraries made of D- and/or L-configuration amino acids, phosphopeptides (including members of random or partially degenerate, directed phosphopeptide libraries), antibodies [e.g. polyclonal, monoclonal, humanized, anti-idiotypic, chimeric, single chain antibodies, fragments, (c.g. Fab, F(ab)<sub>2</sub>, and Fab expression library fragments, and epitope-binding fragments thereof)], and small organic or inorganic molecules. The substance or compound may be an endogenous physiological compound or it may be a natural or synthetic compound.

Substances which modulate a KLK-L Related Protein can be identified based on their ability to bind to a KLK-L Related Protein. Therefore, the invention also provides methods for identifying substances which bind to a KLK-L Related Protein. Substances identified using the methods of the invention may be isolated, cloned and sequenced using conventional techniques. A substance that associates with a polypeptide of the invention may be an agonist or antagonist of the biological or immunological activity of a polypeptide of the invention.

The term "agonist", refers to a molecule that increases the amount of, or prolongs the duration of, the activity of the polypeptide. The term "antagonist" refers to a molecule which decreases the biological or immunological activity of the polypeptide. Agonists and antagonists may include proteins, nucleic acids, carbohydrates, or any other molecules that associate with a polypeptide of the invention.

Substances which can bind with a KLK-L Related Protein may be identified by reacting a KLK-L Related Protein with a test substance which potentially binds to a KLK-L Related Protein, under conditions which permit the formation of substance-KLK-L Related Protein complexes and removing and/or detecting the complexes. The complexes can be detected by assaying for substance-KLK-L Related Protein complexes, for free substance, or for non-complexed KLK-L Related Protein. Conditions which permit the formation of substance-KLK-L Related Protein complexes may be selected having regard to factors such as the nature and amounts of the substance and the protein.

The substance-protein complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against KLK-L Related Protein or the substance, or labeled KLK-L Related Protein, or a labeled substance may be utilized. The antibodies, proteins, or substances may be labeled with a detectable substance as described above.

A KLK-L Related Protein, or the substance used in the method of the invention may be insolubilized. For example, a KLK-L Related Protein, or substance may be bound to a suitable carrier such as agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc. The insolubilized protein or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical

methods, for example, cyanogen bromide coupling.

The invention also contemplates a method for evaluating a compound for its ability to modulate the biological activity of a KLK-L Related Protein of the invention, by assaying for an agonist or antagonist (i.e. enhancer or inhibitor) of the binding of a KLK-L Related Protein with a substance which binds with a KLK-L Related Protein. The basic method for evaluating if a compound is an agonist or antagonist of the binding of a KLK-L Related Protein and a substance that binds to the protein, is to prepare a reaction mixture containing the KLK-L Related Protein and the substance under conditions which permit the formation of substance-KLK-L Related Protein complexes, in the presence of a test compound. The test compound may be initially added to the mixture, or may be added subsequent to the addition of the KLK-L Related Protein and substance. Control reaction mixtures without the test compound or with a placebo are also prepared. The formation of complexes is detected and the formation of complexes in the control reaction but not in the reaction mixture indicates that the test compound interferes with the interaction of the KLK-L Related Protein and substance. The reactions may be carried out in the liquid phase or the KLK-L Related Protein, substance, or test compound may be immobilized as described herein. The ability of a compound to modulate the biological activity of a KLK-L Related Protein of the invention may be tested by determining the biological effects on cells.

It will be understood that the agonists and antagonists i.e. inhibitors and enhancers that can be assayed using the methods of the invention may act on one or more of the binding sites on the protein or substance including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of KLK-L Related Protein with a substance which is capable of binding to the KLK-L Related Protein. Thus, the invention may be used to assay for a compound that competes for the same binding site of a KLK-L Related Protein.

The invention also contemplates methods for identifying compounds that bind to proteins that interact with a KLK-L Related Protein. Protein-protein interactions may be identified using conventional methods such as co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Methods may also be employed that result in the simultaneous identification of genes which encode proteins interacting with a KLK-L Related Protein. These methods include probing expression libraries with labeled KLK-L Related Protein.

Two-hybrid systems may also be used to detect protein interactions *in vivo*. Generally, plasmids are constructed that encode two hybrid proteins. A first hybrid protein consists of the DNA-binding domain of a transcription activator protein fused to a KLK-L Related Protein, and the second hybrid protein consists of the transcription activator protein's activator domain fused to an unknown protein encoded by a cDNA which has been recombined into the plasmid as part of a cDNA library. The plasmids are transformed into a strain of yeast (e.g. *S. cerevisiae*) that contains a reporter gene (e.g. lacZ, luciferase, alkaline phosphatase, horseradish peroxidase) whose regulatory region contains the transcription activator's binding site. The hybrid proteins alone cannot activate the transcription of the reporter gene. However,



interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

It will be appreciated that fusion proteins may be used in the above-described methods. In particular, KLK-L Related Proteins fused to a glutathione-S-transferase may be used in the methods.

The reagents suitable for applying the methods of the invention to evaluate compounds that modulate a KLK-L Related Protein may be packaged into convenient kits providing the necessary materials packaged into suitable containers. The kits may also include suitable supports useful in performing the methods of the invention.

#### 4.3 Compositions and Treatments

The proteins of the invention, substances or compounds identified by the methods described herein, antibodies, and antisense nucleic acid molecules of the invention may be used for modulating the biological activity of a KLK-L Related Protein, and they may be used in the treatment of conditions such as cancer (e.g. prostate, testicular, brain, uterine, thymus, ovarian, colon, ovarian, or breast cancer). Accordingly, the substances, antibodies, peptides, and compounds may be formulated into pharmaceutical compositions for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the active substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The active substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of a pharmaceutical composition of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the substance from the action of enzymes, acids and other natural conditions that may inactivate the substance.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the active substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

Based upon their homology to genes encoding kallikrein, nucleic acid molecules of the invention may be also useful in the treatment of conditions such as hypertension, cardiac hypertrophy, arthritis, inflammatory disorders, neurological disorders, and blood clotting disorders.

Vectors derived from retroviruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids, may be used to deliver nucleic acid molecules to a targeted organ, tissue, or cell population. Methods well known to those skilled in the art may be used to construct recombinant vectors which will express antisense nucleic acid molecules of the invention. (See, for example, the techniques described in Sambrook et al (supra) and Ausubel et al (supra)).

The nucleic acid molecules comprising full length cDNA sequences and/or their regulatory elements enable a skilled artisan to use sequences encoding a protein of the invention as an investigative tool in sense (Yousoufian H and H F Lodish 1993 Mol Cell Biol 13:98-104) or antisense (Eguchi et al (1991) Annu Rev Biochem 60:631-652) regulation of gene function. Such technology is well known in the art, and sense or antisense oligomers, or larger fragments, can be designed from various locations along the coding or control regions.

Genes encoding a protein of the invention can be turned off by transfecting a cell or tissue with vectors which express high levels of a desired KLK-L-encoding fragment. Such constructs can inundate cells with untranslatable sense or antisense sequences. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules until all copies are disabled by endogenous nucleases.

Modifications of gene expression can be obtained by designing antisense molecules, DNA, RNA or PNA, to the regulatory regions of a gene encoding a protein of the invention, ie, the promoters, enhancers, and introns. Preferably, oligonucleotides are derived from the transcription initiation site, eg, between -10 and +10 regions of the leader sequence. The antisense molecules may also be designed so that they block translation of mRNA by preventing the transcript from binding to ribosomes. Inhibition may also be achieved using "triple helix" base-pairing methodology. Triple helix pairing compromises the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Therapeutic advances using triplex DNA were reviewed by Gee J E et al (In: Huber B E and B I Carr (1994) Molecular and Immunologic Approaches, Futura Publishing Co, Mt Kisco N.Y.).

Ribozymes are enzymatic RNA molecules that catalyze the specific cleavage of RNA. Ribozymes act by sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. The invention therefore contemplates engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding a protein of the invention.

Specific ribozyme cleavage sites within any potential RNA target may initially be identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once the sites are identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets

may also be determined by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Methods for introducing vectors into cells or tissues include those methods discussed herein and which are suitable for *in vivo*, *in vitro* and *ex vivo* therapy. For *ex vivo* therapy, vectors may be introduced into stem cells obtained from a patient and clonally propagated for autologous transplant into the same patient (See U.S. Pat. Nos. 5,399,493 and 5,437,994). Delivery by transfection and by liposome are well known in the art.

The nucleic acid molecules disclosed herein may also be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including but not limited to such properties as the triplet genetic code and specific base pair interactions.

The invention also provides methods for studying the function of a polypeptide of the invention. Cells, tissues, and non-human animals lacking in expression or partially lacking in expression of a nucleic acid molecule or gene of the invention may be developed using recombinant expression vectors of the invention having specific deletion or insertion mutations in the gene. A recombinant expression vector may be used to inactivate or alter the endogenous gene by homologous recombination, and thereby create a deficient cell, tissue, or animal.

Null alleles may be generated in cells, such as embryonic stem cells by deletion mutation. A recombinant gene may also be engineered to contain an insertion mutation that inactivates the gene. Such a construct may then be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, electroporation, injection etc. Cells lacking an intact gene may then be identified, for example by Southern blotting, Northern Blotting, or by assaying for expression of the encoded polypeptide using the methods described herein. Such cells may then be fused to embryonic stem cells to generate transgenic non-human animals deficient in a polypeptide of the invention. Germline transmission of the mutation may be achieved, for example, by aggregating the embryonic stem cells with early stage embryos, such as 8 cell embryos, *in vitro*; transferring the resulting blastocysts into recipient females and; generating germline transmission of the resulting aggregation chimeras. Such a mutant animal may be used to define specific cell populations, developmental patterns and *in vivo* processes, normally dependent on gene expression.

The invention thus provides a transgenic non-human mammal all of whose germ cells and somatic cells contain a recombinant expression vector that inactivates or alters a gene encoding a KLK-L Related Protein. In an embodiment the invention provides a transgenic non-human mammal all of whose germ cells and somatic cells contain a recombinant expression vector that inactivates or alters a gene encoding a KLK-L Related Protein resulting in a KLK-L Related Protein associated pathology. Further the invention provides a transgenic non-human mammal which does not express a KLK-L Related Protein of the invention. In an embodiment, the invention provides a transgenic non-human mammal which does not express a KLK-L Related Protein of the invention resulting in a KLK-L Related Protein associated pathology. A KLK-L Related Protein pathology refers to a phenotype observed for a KLK-L Related Protein homozygous mutant.

A transgenic non-human animal includes but is not limited to mouse, rat, rabbit, sheep, hamster, dog, guinea pig, micro-pig, pig, cat, goat, and non-human primates, preferably mouse.

The invention also provides a transgenic non-human animal assay system which provides a model system for testing for an agent that reduces or inhibits a pathology associated with an KLK-L Related Protein, preferably a KLK-L Related Protein associated pathology, comprising:

- (a) administering the agent to a transgenic non-human animal of the invention; and
- (b) determining whether said agent reduces or inhibits the pathology (e.g. KLK-L Related Protein associated pathology) in the transgenic non-human animal relative to a transgenic non-human animal of step (a) which has not been administered the agent.

The agent may be useful in the treatment and prophylaxis of conditions such as cancer as discussed herein. The agents may also be incorporated in a pharmaceutical composition as described herein.

The activity of the proteins, substances, compounds, antibodies, nucleic acid molecules, agents, and compositions of the invention may be confirmed in animal experimental model systems. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or with experimental animals, such as by calculating the  $ED_{50}$  (the dose therapeutically effective in 50% of the population) or  $LD_{50}$  (the dose lethal to 50% of the population) statistics. The therapeutic index is the dose ratio of therapeutic to toxic effects and it can be expressed as the  $ED_{50}/LD_{50}$  ratio. Pharmaceutical compositions which exhibit large therapeutic indices are preferred.

The following non-limiting examples are illustrative of the present invention:

## Examples

### Example 1

#### MATERIALS AND METHODS

##### Identification of positive PAC and BAC genomic clones from a human genomic DNA library

The sequence of PSA, KLK1, KLK2, NES1 and Zyme genes is already known. Polymerase chain reaction (PCR)-based amplification protocols have been developed which allowed generation of PCR products specific for each one of these genes. Using these PCR products as probes, labeled with  $^{32}P$ , a human genomic DNA PAC library and a human genomic DNA BAC library was screened for the purpose of identifying positive clones of approximately 100-150 Kb long. The general strategies for these experiments have been published elsewhere (14). The genomic libraries were spotted in duplicate on nylon membranes and positive clones were further confirmed by Southern blot analysis as described (14).

##### DNA sequences on chromosome 19

The Lawrence Livermore National Laboratory participates in the sequencing of the human genome project and focuses on sequencing chromosome 19. Large sequencing information on this chromosome is available at the website of the Lawrence Livermore National Laboratory (<http://www-bio.llnl.gov/genome/gemnome.html>).

Approximately 300 Kb of genomic sequences were obtained from that website, encompassing a region on chromosome 19q13.3 - 13.4, where the known kallikrein genes are localized. This 300 Kb of sequence is represented by 8 contigs of variable lengths. By using a number of different computer

programs, an almost contiguous sequence of the region was established as shown diagrammatically in Figure 1 and Figure 28. Some of the contigs were reversed as shown in Figure 1 in order to reconstruct the area on both strands of DNA.

By using the published sequences of PSA, KLK2, NES1 and Zyme and the computer software BLAST 2, using alignment strategies, the relative positions of these genes on the contiguous map were identified (Figure 28). These known genes served as hallmarks for further studies. An EcoRI restriction map of the area is also available at the website of the Lawrence Livermore National Laboratory. Using this restriction map and the computer program WebCutter (<http://www.firstmarket.com/cutter/cut2.html>), a restriction study analysis of the available sequence was performed to further confirm the assignment and relative positions of these contigs along chromosome 19. The obtained configuration and the relative location of the known genes are presented in Figure 1.

#### **Gene prediction analysis**

For exon prediction analysis of the whole genomic area, a number of different computer programs were used. All the programs were initially tested using known genomic sequences of the PSA, Zyme, and NES1 genes. The more reliable computer programs, GeneBuilder (gene prediction), GeneBuilder (exon prediction), Graal 2 and GENEID-3 were selected for further use.

#### **Protein homology searching**

Putative exons of the new genes were first translated to the corresponding amino acid sequences. BLAST homology searching for the proteins encoded by the exons of the putative new genes were performed using the BLASTP program and the Genbank databases.

### **RESULTS**

#### **Relative position of PSA, KLK2, Zyme and NES1 on Chromosome 19**

Screening of the human BAC library identified two clones which were positive for the Zyme gene (clones BAC 288H1 and BAC 76F7). These BACs were further analyzed by PCR and primers specific for PSA, NES1, KLK1 and KLK2. These analyses indicated that both BACs were positive for Zyme, PSA and KLK2 and negative for KLK1 and NES1 genes.

Screening of the human PAC genomic library identified a PAC clone which was positive for NES1 (clone PAC 34B1). Further PCR analysis indicated that this PAC clone was positive for NES1 and KLK1 genes and negative for PSA, KLK2 and Zyme. Combination of this information with the EcoRI restriction map of the region allowed establishment of the relative positions of these four genes. PSA is the most centromeric, followed by KLK2, Zyme and NES1. Further alignment of the known sequences of these genes with the 300 Kb contig enabled precise localization of all four genes and determination of the direction of transcription, as shown by the arrows in Figure 1. The KLK1 gene sequence was not identified on any of these contigs and appears to be further telomeric to NES1 (since it is co-localized on the same PAC as NES1).

#### **Identification of new genes**

A set of rules was used to consider the presence of a new gene in the genomic area of interest as follows:

1. Clusters of at least 3 exons should be found.
2. Only exons with high prediction score ("good" or "excellent" quality, as indicated by the searching programs) were considered for the construction of the putative new genes.
3. Exons predicted were reliable only if they were identified by at least two different exon prediction programs.

By using this strategy, eleven putative new genes were identified of which three were found on subsequent homology analysis to be known genes not previously mapped i.e. the human stratum corneum chymotrypsin enzyme (HSCCE), human neuropsin, and trypsin-like serine protease (TLSP). Their relative location is shown in Figure 1. The five genes all have variable homologies with known human or animal kallikrein proteins and/or other known serine proteases (depicted as KLK-L1, KLK-L2, KLK-L3, KLK-L4 and KLK-L5 in Figure 1 and KLK-L1 to KLK-L6 in Figure 28).

In Tables 1 to 5, the preliminary exon structure and partial protein sequence for each one of the newly identified genes is shown. In Table 6, some proteins are presented which appear, on preliminary analysis, to be homologous to the proteins encoded by the putative new genes. SEQ. ID. NOs. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, and 67 show amino acid sequences of KLK-L1-KLK-L6, and SEQ. ID. NOs. 1, 13, 21, 43, 56, and 65 show nucleic acid sequences of the genes encoding KLK-L1- KLK-L6.

## DISCUSSION

Prediction of protein-coding genes in newly sequenced DNA becomes very important after the establishment of large genome sequencing projects. This problem is complicated due to the exon-intron structure of the eukaryotic genes which interrupts the coding sequence in many unequal parts. In order to predict the protein-coding exons and overall gene structure, a number of computer programs were developed. All these programs are based on the combination of potential functional signals with the global statistical properties of known protein-coding regions (15). However, the most powerful approach for gene structure prediction is to combine information about potential functional signals (splice sites, translation start or stop signal etc.) together with the statistical properties of coding sequences (coding potential) along with information about homologies between the predicted protein and known protein families (16).

In mouse and rat, kallikreins are encoded by large multigene families and these genes tend to cluster in groups with a distance as small as 3.3 – 7.0 Kb (3). A strong conservation of gene order between human chromosome 19q13.1 – q13.4 and 17 loci in a 20-cM proximal part of mouse chromosome 7, including the kallikrein locus, has been documented (17).

In humans, only a few kallikrein genes were identified. In fact, only KLK1, KLK2 and KLK3 (PSA) are considered to represent the human kallikrein gene family (9). The work described herein provides strong evidence that a large number of kallikrein-like genes are clustered within a 300Kb region around chromosome 19q13.2 – q13.4. The three established human kallikreins (KLK1, KLK2, KLK3), Zyme and NES1, as well as the stratum corneum chymotryptic enzyme, neuropsin, and TLSP (trypsin-like serine protease) and another five new genes, KLK-L1 to KLK-L5, may constitute a large gene family. This will bring the total number of kallikrein or kallikrein-like genes in this region of chromosome 19 to thirteen.

The human stratum corneum chymotryptic enzyme (19), neuropsin (20) and trypsin-like serine

protease (TLSP) (21) are three previously characterized genes which have many structural similarities with the kallikreins and other members of the serine protease family. However, they have not been mapped in the past. Their precise mapping in the region of the kallikrein gene family indicates that these three genes, along with the ones that were newly identified, or are already known, constitute a family that likely originated by duplication of an ancestral gene. The relative localization of all these genes is depicted in Figure 1.

Kallikrein genes are a subfamily of serine proteases, traditionally characterized by their ability to liberate lysyl-bradykinin (kallidin) from kininogen (18). More recently, however, a new, structural concept has emerged to describe kallikreins. From accumulated sequence data, it is now clear that the mouse has many genes with high homology to kallikrein coding sequences (19-20). Richard and co-workers have contributed to the concept of a "kallikrein multigene family" to refer to these genes (21-22). This definition is not based much on specific enzymatic function of the gene product, but more on its sequence homology and their close linkage on mouse chromosome 7. In humans, only KLK1 meets the functional definition of a kallikrein. KLK2 has trypsin-like enzymatic activity and KLK3 (PSA) has very weak chymotrypsin-like enzymatic activity. These activities of KLK2 and KLK3 are not known to liberate biologically active peptides from precursors. Based on the newer definition, members of the kallikrein family include, not only the gene for the kallikrein enzyme, but also genes encoding other homologous proteases, including the enzyme that processes the precursors of the nerve growth factor and epidermal growth factor (8). Therefore, it is important to note the clear distinction between the enzyme kallikrein and a kallikrein or a kallikrein-like gene.

In carrying out the study only exons were considered which were predicted with "good" or "excellent" quality and only exons were considered which were predicted by at least two different programs. Moreover, the presence of a putative gene was only considered when at least three exons clustered coordinately in that region. Additional evidence that these new genes are indeed homologous to the known kallikreins and other serine proteases comes from comparison of the intron phases. As published previously (14), trypsinogen, PSA and NES1 have 5 coding exons of which the first has intron phase I (the intron occurs after the first nucleotide of the codon), the second has intron phase II (the intron occurs after the second nucleotide and the codon), the third has intron phase I and the fourth has intron phase 0 (the intron occurs between codons). The fifth exon contains the stop codon. The intron phases of the predicted new kallikrein-like genes follow these rules and are shown in the respective tables. Further support comes from the identification in the new genes, of the conserved amino acids of the catalytic domain of the serine proteases, as presented in Tables 1 - 5.

In order to test the accuracy of the computer programs, known genomic areas containing the PSA, Zyme and KLK2 genes were tested. Two of these programs (Grail 2 and GeneBuilder) were able to detect about 95% of the tested known genes. Matches with expressed sequence tag sequences (EST) can also be employed for gene structure prediction in the GeneBuilder program and this can significantly improve the power of the program especially at high stringency (e.g. >95% homology).

In mouse, ten of the kallikrein genes appear to be pseudogenes (9).

## Example 2

### PROSTASE/KLK-L1 in prostate and breast tissues

The fine mapping of the prostase/KLK-L1 gene and its chromosomal localization in relation to a number of other homologous genes also mapping to the same region are described. In addition, extensive tissue expression studies were carried out that demonstrate that, in addition to prostate (which shows the highest expression), that prostase/KLK-L1 is also expressed in female breasts, testis, adrenals, uterus, colon, thyroid, brain, spinal cord and salivary glands. Furthermore, the gene is up-regulated by androgens and progestins in the breast carcinoma cell line BT-474.

### Materials and Methods

#### 10 DNA sequences on chromosome 19

Large DNA sequencing data for chromosome 19 is available at the web site of the Lawrence Livermore National Laboratory (LLNL) (<http://www-bio.llnl.gov/genome/genome.html>). Approximately 300 Kb of genomic sequence was obtained from that web site, encompassing a region on chromosome 19q13.3 - 13.4, where the known kallikrein genes are localized. This sequence is represented by 9 contigs of variable lengths. By using the sequences of PSA, KLK2, NES1 and protease M and the alignment program BLAST 2 (37), the relative positions of these genes on the contiguous map were located.

#### Gene prediction analysis

For exon prediction analysis of the whole genomic area, a number of different computer programs were used. Originally all these programs were tested using the known genomic sequences of the PSA, protease M and NES1 genes. The most reliable computer programs GeneBuilder (gene prediction) [<http://l25.itba.mi.cnr.it/~webgene/genebuilder.html> ] GeneBuilder (exon prediction) [<http://l25.itba.mi.cnr.it/~webgene/genebuilder.html> ], Grail 2 [<http://compbio.ornl.gov> ], and GENEID-3 [<http://apollo.imim.es/geneid.html>] were selected for further use.

#### Protein homology searching

Putative exons of the newly identified gene were first translated to the corresponding amino acid sequences. BLAST homology searching for the proteins encoded by the exons were performed using the BLASTP program and the GenBank databases (37).

#### Searching expressed sequence tags (ESTs)

Sequence homology searching was performed using the BLASTN algorithm (37) on the National Center for Biotechnology Information web server (<http://www.ncbi.nlm.nih.gov/BLAST/>) against the human EST database (dbEST). Clones with > 95% homology were obtained from the I.M.A.G.E. (38) consortium through Research Genetics Inc, Huntsville, AL and from The Institute for Genomic Research (TIGR) (<http://WWW.TIGR.ORG/tldb/tdb.html>) (Table 7). Clones were propagated, purified and then sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

#### 35 Breast cancer cell line and stimulation experiments

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Rockville, MD. BT-474 cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%),



antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of  $10^{-8}$  M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for mRNA extraction.

#### Reverse transcriptase polymerase chain reaction

Total RNA was extracted from the breast cancer cells using Trizol reagent (Gibco BRL) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2 µg of total RNA was reverse transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20 µl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (Table 8). PCR was carried out in a reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 minutes to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute and a final extension at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

#### Tissue expression of KLK-L1

Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA. cDNA was prepared as described above for the tissue culture experiments and used for PCR reactions with the primers described in Table 8 (SEQ. ID. Nos 5-12). Tissue cDNAs were amplified at various dilutions.

#### Cloning and sequencing of the PCR products

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, by an automated DNA sequencer.

#### Results

#### Identification of the prostatic/PLK-L1 gene

The exon prediction strategy of the 300Kb DNA sequences around chromosome 19q13.3 - q13.4 identified a novel gene with a structure reminiscent of a serine protease. The major features of this gene were its homology, at the amino acid and DNA level, with other human kallikrein genes; the conservation of the catalytic triad (histidine, aspartic acid, and serine), the number of exons and the complete conservation of the intron phases.

#### EST sequence homology search

EST sequence homology search of the putative exons obtained from the gene prediction programs (as described above) against the human EST database (dbEST) revealed five expressed sequence tags

(ESTs) with >95 % identity to the putative exons of the gene (Table 7). Positive clones were obtained and the inserts were sequenced from both directions. Alignment was used to compare between the EST sequences and the exons predicted by the computer programs, and final selection of the exon-intron splice sites was made according to the EST sequences. Furthermore, many of the ESTs were overlapping, further ensuring the accuracy of the data.

The coding sequence of the klk-L2 gene is shown in SEQ. ID. NO. 1 and GenBank Accession # AF135023. The exons of the gene are as follows: exon 1 (939-999); exon 2 (2263-2425); exon 3 (2847-3097); exon 4 (3181-3317); and exon 5 (4588-4740). The amino acid sequence of KLK-L2 proteins are shown in SEQ. ID. Nos. 2 and 3.

#### Mapping and chromosomal localization of prostate /KLK-L1 gene

Alignment of the prostate/ KLK-L1 sequence and the sequences of other known kallikrein genes within the 300 Kb area of the contigs constructed at the Lawrence Livermore National Laboratory enabled precise localization of all genes and to determine the direction of transcription, as shown in Figure 2. The distance between PSA and KLK2 genes was calculated to be 12,508 bp. The prostate/KLK-L1 gene is 26,229 bp more telomeric to KLK2 and transcribes in the opposite direction. The zyme gene is about 51 Kb more telomeric to the prostate gene and transcribes in the same direction. The human stratum corneum chymotryptic enzyme gene, the neuropsin gene and the NES 1 gene are all further telomeric to zyme and all transcribe in the same direction as zyme.

#### Tissue expression of the prostate/KLK-L1 gene

The tissues that express the prostate/KLK-L1 gene were assessed by RT-PCR. The experiments were performed at various dilutions of the cDNAs to obtain some information about the relative levels of expression. RT-PCR for actin was used as a positive control and RT-PCR for the PSA cDNA was used as another positive control with tissue restricted specificity. Positive ESTs for prostate/KLK-L1 were used as controls for the PCR procedure. The PSA gene was found to be highly expressed in the prostate, as expected, and to a lower extent in mammary and salivary glands as also expected from recent literature reports (24, 25). Very low expression of PSA in the thyroid gland, trachea and testis was also found, a finding that accords with recent RT-PCR data by others (26).

The tissue expression of prostate/KLK-L1 is summarized in Table 9 and Figure 3. This protease is primarily expressed in the prostate, testis, adrenals, uterus, thyroid, colon, central nervous system and mammary tissues, and, at much lower levels in other tissues. The specificity of the RT-PCR procedure was verified for prostate/KLK-L1 by cloning the PCR products from mammary, testicular and prostate tissues and sequencing them. One example with mammary tissue is shown in Figure 4. All cloned PCR products were identical in sequence to the cDNA sequence reported for the prostate/KLK-L1.

#### Hormonal regulation of the prostate/KLK-L1 gene

The steroid hormone receptor-positive breast carcinoma cell line BT-474 was used as a model system to evaluate whether prostate/KLK-L1 expression is under steroid hormone regulation. As shown in Figure 5, the controls worked as expected i. e., actin positivity without hormonal regulation in all cDNAs, only estrogen up-regulation of the pS2 gene and up-regulation of the PSA gene by androgens and

progestins. Prostase/KLK-L1 is up-regulated primarily by androgens and progestins, similarly to PSA. This up-regulation was dose-dependent and it was evident at steroid hormone levels  $\geq 10^{-10}$  M.

## DISCUSSION

The KLK3 gene encodes for PSA, a protein that currently represents the best tumor marker available (24). Since in rodents there are so many kallikrein genes, the restriction of this family to only 3 genes in humans was somewhat surprising. More recently, new candidate kallikrein genes in humans have been discovered, including NES1 (13) and zyme/protease M/neurosin (10-12). The known kallikreins and the newly discovered kallikrein-like genes share the following similarities: (a) they encode serine proteases (b) they have five coding exons (c) they share significant DNA and protein homologies with each other (d) they map in the same locus on chromosome 19q13.3-q13.4, a region that is structurally similar to an area on mouse chromosome 7, where all the mouse kallikrein genes are localized (e) they appear to be regulated by steroid hormones. Prostase/KLK-L1 is a member of the same family since these common characteristics are also shared by the newly discovered gene.

The exact localization of the KLK-L1 gene and its position in relation to other genes in the area (Figure 2) was determined. Prostase/KLK-L1 lies between KLK2 and zyme.

Irwin et al. (27) have proposed that the serine protease genes can be classified into five different groups according to intron position. The established kallikreins (KLK1, KLK2, and PSA), trypsinogen and chymotrypsinogen belong to a group that has: (1) an intron just downstream from the codon for the active site histidine residue, (2) a second intron downstream from the exon containing the codon for the active site aspartic acid residue, and (3) a third intron just upstream from the exon containing the codon for the active site serine residue. As seen in Figure 6, the genomic organization of prostase/KLK-L1 gene is very similar to this group of genes. The lengths of the coding parts of exons 1-5 are 61, 163, 263, 137 and 153 bp, respectively, which are close or identical to the lengths of the exons of the kallikrein genes and also, similar or identical to those of other newly discovered genes in the same chromosomal region like the NES1 (14), zyme/protease M/neurosin (10-12) and neuropsin (28) genes.

The sensitive RT-PCR protocol reveals that the KLK-L1 enzyme is expressed in prostatic tissue and it is also expressed in significant amounts in other tissues, including testis, female mammary gland, adrenals, uterus, thyroid, colon, brain, lung and salivary glands (Figure 3 and Table 9). The specificity of the RT-PCR primers was verified by sequencing the obtained PCR products, with one example shown in Figure 4 (SEQ.ID.NO. 4). Tissue culture studies with the breast carcinoma cell line BT-474 further confirm not only the ability of these cells to produce prostase/KLK-L1 but also its hormonal regulation (Figure 5).

An interesting theme is now developing involving the group of homologous genes on chromosome 19q13.3 (PSA, KLK2, prostase, zyme, and NES1). The combined data suggest that all of them are expressed in prostate and breast tissues, and all of them are hormonally regulated. All these genes may be part of a cascade pathway that plays a role in cell proliferation, differentiation or apoptosis by regulating (positively or negatively) growth factors or their receptors or cytokines, through proteolysis (30). Also interesting is the linkage of locus 19q13 to solid tumors and gliomas (31) which raises the possibility that some of the genes in the region may be disrupted by rearrangements.

The KLK-1L gene encodes for a serine protease that shows homology with other members of the kallikrein gene family and maps to the same chromosomal location. Many structural features of the kallikreins are conserved in prostate/KLK-L1. The precise mapping of this gene between the two known genes KLK2 and zyme is presented. It is further demonstrated that prostate/KLK-L1 is expressed in many tissues, in addition to the prostate, including the female breast. This gene is also herein referred to as 'protease'. It has been further demonstrated, using breast carcinoma cell lines, that prostate/KLK-L1 can be produced by these cells and that its expression is significantly up-regulated by androgens and progestins. Based on information for other homologous genes in the area (PSA, zyme, and NES1), prostate/KLK-L1 may be involved in the pathogenesis and/or progression of prostate, breast and possibly other cancers.

### Example 3

## IDENTIFICATION OF THE KLK-L2 GENE

### Materials and Methods

#### DNA sequence on chromosome 19

Sequencing data of approximately 300Kb of nucleotides on chromosome 19q13.3-q13.4 was obtained from the web site of the Lawrence Livermore National Laboratory (LLNL) (<http://www.bio.llnl.gov/genome/genome.html>). This sequence was in the form of 9 contigs of different lengths. A restriction analysis study of the available sequences was performed using the "WebCutter" computer program (<http://www.firstmarket.com/cutter/cu2.html>) and with the aid of the EcoRI restriction map of this area (also available from the LLNL web site) an almost contiguous stretch of genomic sequences was constructed. The relative positions of the known kallikrein genes: PSA (GenBank accession # X14810), KLK2 (GenBank accession # M18157), and zyme (GenBank accession # U60801) was determined using the alignment program BLAST 2 (37).

#### New Gene Identification

A number of computer programs were used to predict the presence of putative new genes in the genomic area of interest. These programs were initially tested using the known genomic sequences of the PSA, protease M and NES1 genes. The most reliable computer programs GeneBuilder (gene prediction) (<http://l25.itba.mi.cnr.it/~webgene/genebuilder.html>) GeneBuilder (exon prediction) (<http://l25.itba.mi.cnr.it/~webgene/genebuilder.html>), Grail 2 (<http://compbio.ornl.gov>) and GENEID-3 (<http://apollo.imm.cs/genid.html>) were selected for further use.

#### Expressed sequence tag (EST) searching

The predicted exons of the putative new gene were subjected to homology search using the BLASTN algorithm (37) on the National Center for Biotechnology Information web server (<http://www.ncbi.nlm.nih.gov/BLAST/>) against the human EST database (dbEST). Clones with > 95% homology were obtained from the I.M.A.G.E. consortium (38) through Research Genetics Inc, Huntsville, AL (Table 10). The clones were propagated, purified and sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

#### Rapid amplification of cDNA ends (5' RACE)

According to the EST sequence data and the predicted structure of the gene, two gene-specific primers were designed (R1 & R2) (Table 11). Two rounds of RACE reactions (nested PCR) were performed with 5µl Marathon Ready™ cDNA of human testis (Clontech, Palo Alto, CA, USA) as a template. The reaction mix and PCR conditions were conducted according to the manufacturer's recommendations. In brief, denaturation was done for 5 min at 94°C followed by 94°C for 5 sec followed by 72°C for 2 min for 5 cycles, then 94°C for 5 sec followed by 70°C for 2 min for 5 cycles then 94°C for 5 sec followed by 65°C for 2 min for 30 cycles for the first reaction and 25 cycles for the nested PCR reaction.

#### **Tissue expression**

Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA. cDNA was prepared as described below for the tissue culture experiments and used for PCR reactions with the primers described in Table 11 (SEQ. ID. Nos 9-12, 15-20). Tissue cDNAs were amplified at various dilutions.

#### **Breast cancer cell line and hormonal stimulation experiments**

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of  $10^{-8}$  M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for mRNA extraction.

#### **Reverse transcriptase polymerase chain reaction**

Total RNA was extracted from the breast cancer cells using Trizol reagent (Gibco BRL) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2 µg of total RNA was reverse-transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20 µl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (Table 11) and PCR was carried out in a reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 minutes to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute and a final extension at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

#### Structure analysis

Multiple alignment was performed using the Clustal X software package available at: <ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx/> (clustalx1.64b.msw.exe) and the multiple alignment program available from the Baylor College of Medicine (BCM), Houston, TX, USA ([kiwi.imgen.bcm.tmc.edu:8808/search-launcher/launcher/html](http://www.kiwimc.tmc.edu:8808/search-launcher/launcher.html)). Phylogenetic studies were performed using the Phylip software package available at: <http://evolution.genetics.washington.edu/phylip/getme.html>. Distance matrix analysis was performed using the "Neighbor-Joining/UPGMA" program and parsimony analysis was done using the "Protpars" program. Hydrophobicity study was performed using the BCM search launcher programs (<http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html>). Signal peptide was predicted using the "SignalP" server (<http://www.cbs.dtu.dk/services/signal>). Protein structure analysis was performed by "SAPS" (structural analysis of protein sequence) program (<http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html>).

#### RESULTS

Computer analysis of the genomic sequence predicted a putative new gene consisting of four exons. This gene was detected by all programs used and all exons had high prediction scores. EST sequence homology search of the putative exons against the human EST database (dbEST) revealed nine expressed sequence tag (EST) clones from different tissues with >95 % identity to the putative exons of the gene (Table 10). Positive clones were obtained and the inserts were sequenced from both directions. The "Blast 2 sequences" program was used to compare the EST sequences with the predicted exons, and final selection of the exon-intron splice sites was done according to the EST sequences. The presence of many areas of overlap between the various EST sequences allowed further verification of the structure of the new gene. The coding sequence of the gene is shown in SEQ. ID. NO. 13 and GenBank Accession #AF135028. The 3' end of the gene was verified by the presence of poly A stretches that are not present in the genomic sequence at the end of two of the sequenced ESTs. One of the sequenced ESTs revealed the presence of an additional exon at the 5' end. The nucleotide sequence of this exon matches exactly with the genomic sequence. To further identify the 5' end of the gene, 5' RACE was performed but no additional sequence could be obtained. However, as is the case with other kallikreins, the presence of further upstream untranslated exon(s) could not be excluded. The amino acid sequence of KLK-L2 is shown in SEQ. ID. No. 14.

#### Mapping and chromosomal localization of the KLK-L2 gene

Alignment of KLK-L2 gene and the sequences of other known kallikrein genes within the 300 Kb area of interest enabled precise localization of all genes and determination of the direction of transcription, as shown by the arrows in Figure 8. The PSA gene was found to be the most centromeric, separated by 12,508 base pairs (bp) from KLK2, and both genes are transcribed in the same direction (centromere to telomere). The prostate/KLK-L1 gene is 26,229 bp more telomeric and transcribes in the opposite

direction, followed by KLK-L2. The distance between KLK-L1 and KLK-L2 is about 35 Kilobases (Kb). The zyme gene is 5,981 bp more telomeric and the latter 3 genes are all transcribed in the same direction (Figure 8).

#### Structural characterization of the KLK-L2 gene and its protein product

5 The KLK-L2 gene, as presented in Figure 7, is formed of 5 coding exons and 4 intervening introns, spanning an area of 9,349 bp of genomic sequence on chromosome 19q13.3-q13.4. The lengths of the exons are 73, 262, 257, 134, and 156 bp, respectively. The intron/exon splice sites (mGT...AGm) and their flanking sequences are closely related to the consensus splicing sites (-mGTAAGT ...CAGm-) (32). The presumptive protein coding region of the KLK-L2 gene is formed of 879 bp nucleotide sequence encoding  
10 a deduced 293-amino acid polypeptide with a predicted molecular weight of 32 KDa. There are two potential translation initiation codons (ATG) at positions 1 and 25 of the predicted first exon (numbers refer to SEQ. ID. NO. 13 and GenBank Accession #AF135028). It is assumed that the first ATG will be the initiation codon, since : (1) the flanking sequence of that codon (GCGGCCATGG) matches closely with the Kozak consensus sequence for initiation of translation (GCC A/G CCATGG) (33) and is exactly the  
15 same as that of the homologous zyme gene. At this initiation codon, the putative signal sequence at the N-terminus is similar to other trypsin-like serine proteases (prostase and EMSP) (Figure 9). The cDNA ends with a 328 bp of 3' untranslated region containing a conserved polyadenylation signal (AATAAA) located 11 bp up-stream of the poly A tail ( at a position exactly the same as that of the zyme poly A tail)(11).

A hydrophobicity study of the KLK-L2 gene shows a hydrophobic region in the N-terminal region  
20 of the protein (Figure 10), suggesting that a presumed signal peptide is present. By computer analysis, a 29-amino acid signal peptide is predicted with a cleavage site at the carboxyl end of Ala<sup>29</sup>. For better characterization of the predicted structural motif of the KLK-L2 protein, it was aligned with other members of the kallikrein multi-gene family, (Figure 9), and the predicted signal peptide cleavage site was found to match with the predicted signal cleavage sites of zyme (11), KLK1(1), KLK2 (8), and KLK-L1. Also,  
25 sequence alignment supports, by analogy, the presence of a cleavage site at the carboxyl end of Ser<sup>66</sup>, which is the exact site predicted for cleavage of the activation peptide of all the other kallikreins aligned in Figure 9. Interestingly, the starting amino acid sequence of the mature protein (I I N G (S) D C ) is conserved in the prostase and enamel matrix serine proteinase 1 (EMSP) genes. Thus, like other kallikreins, KLK-L2 is likely also synthesized as a preproenzyme that contains an N-terminal signal peptide (prezymogen)  
30 followed by an activation peptide and the enzymatic domain.

The presence of aspartate (D) in position 239 suggests that KLK-L2 will possess a trypsin-like cleavage pattern like most of the other kallikreins (e.g., KLK1, KLK2, TLSP, neuropsin, zyme, prostase, and EMSP) but different from PSA which has a serine (S) residue in the corresponding position, and is known to have a chymotrypsin like activity (Figure 9). The dotted region in Figure 9 indicates an 11-amino  
35 acid loop characteristic of the classical kallikreins (PSA, KLK1, and KLK2) but not found in KLK-L2 or other members of the kallikrein-like gene family (11).

#### Homology with the kallikrein multi-gene family

The mature 227-amino acid sequence of the predicted protein was aligned against the GenBank

database and the known kallikreins using the "BLASTP" and "BLAST 2 sequence" programs. KLK-L2 is found to have 54% amino acid sequence identity and 68% similarity with the enamel matrix serine proteinase 1 (EMSP1) gene, 50% identity with both trypsin like serine protease (TLSP) and neuropsin genes and 47%, 46%, and 42% identity with trypsinogen, zyme, and PSA genes, respectively. The multiple alignment study shows that the typical catalytic triad of serine proteases is conserved in the KLK-L2 gene (H<sup>108</sup>, D<sup>153</sup>, and S<sup>245</sup>) and, as the case with all other kallikreins, a well conserved peptide motif is found around the amino acid residues of the catalytic triad [i.e., histidine (WLLTAAHC), serine(GDSGGP), and aspartate(DLMLI)] (10, 11).

Twelve cysteine residues are present in the putative mature KLK-L2 protein, ten of them are conserved in all the serine proteases that are aligned in Figure 9, and would be expected to form disulphide bridges. The other two cysteines (C<sup>178</sup> and C<sup>279</sup>) are not found in PSA, KLK1, KLK2 or trypsinogen, however, they are found in similar positions in prostase, EMSP1, zyme, neuropsin, and TLSP genes and are expected to form an additional disulphide bond. Twenty nine "invariant" amino acids surrounding the active site of serine proteases have been described (39). Of these, twenty-six are conserved in KLK-L2. One of the non-conserved amino acids (Ser<sup>210</sup> instead of Pro) is also found in prostase and EMSP1 genes, the second (Leu<sup>603</sup> instead of Val) is also found in TLSP gene, and the third (Val<sup>174</sup> instead of Leu) is also not conserved in prostase or EMSP1 genes. According to protein evolution studies, each of these amino acid changes represents a conserved evolutionary substitution to a protein of the same group (39).

#### Evolution of the KLK-L2 gene

To predict the phylogenetic relatedness of the KLK-L2 gene with other serine proteases, the amino acid sequences of the kallikrein genes were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-joining/UPGMA method (Figure 10). Phylogenetic analysis separated the classical kallikreins (KLK1, KLK2, and PSA) and grouped the KLK-L2 with KLK-L1, EMSP1, and TLSP (40, 41).

#### Tissue expression of the KLK-L2 gene

As shown in Table 12 and Figure 11, the KLK-L2 gene is primarily expressed in the brain, mammary gland, and testis but lower levels of expression are found in many other tissues. In order to verify the RT-PCR specificity, the PCR products were cloned and sequenced.

#### Hormonal regulation of the KLK-L2 gene

A steroid hormone receptor positive breast cancer cell line (BT-474) was used as a model to verify whether the KLK-L2 gene is under steroid hormone regulation. PSA was used as a control known to be upregulated by androgens and progestins and pS2 as an estrogen upregulated control. The results indicate that KLK-L2 is up-regulated by estrogens and progestins (Figure 12).

#### Expression of KLK-L2 in Ovarian Tissues

KLK-L2 is up-regulated (overexpressed) in ovarian tumors (Figure 13).

#### Discussion

With the aid of computer programs for gene prediction and the available EST database, a new gene, named KLK-L2 (for kallikrein like gene 2) was identified. The 3' end of the gene was verified by the



presence of "poly A" stretches in the sequenced ESTs which were not found in the genomic sequence, and the start of translation was identified by the presence of a start codon in a well conserved consensus Kozak sequence.

As is the case with other kallikreins, the KLK-L2 gene is composed of 5 coding exons and 4  
5 intervening introns and, except for the second coding exon, the exon lengths are comparable to those of other members of the kallikrein gene family (Figure 6). The exon-intron splice junctions were identified by comparing the genomic sequence with the EST sequence and were further confirmed by the conservation of the consensus splice sequence (-mGT.....AGm-) (32), and the fully conserved intron phases, as shown in Figure 6. Furthermore, the position of the catalytic triad residues in relation to the different exons is also  
10 conserved (Figure 6). As is the case with most other kallikreins, except PSA and HSCCE, KLK-L2 is more functionally related to trypsin than to chymotrypsin (34). The wide range of tissue expression of KLK-L2 should not be surprising since, by using the more sensitive RT-PCR technique instead of Northern blot analysis, many kallikrein genes were found to be expressed in a wide variety of tissues including salivary  
15 gland, kidney, pancreas, brain, and tissues of the reproductive system (uterus, mammary gland, ovary, and testis) (34). KLK-L2 is highly expressed in the brain. Another kallikrein, neuropsin, was also found to be highly expressed in the brain and has been shown to have important roles in neural plasticity in mice (35). Also, the zyme gene is highly expressed in the brain and appears to have amyloidogenic potential (11). Taken together, these data point to a possible role of KLK-L2 in the central nervous system.

It was initially thought that each kallikrein enzyme has one specific physiological substrate.  
20 However, the increasing number of substrates, which purified proteins can cleave *in vitro*, has led to the suggestion that they may perform a variety of functions in different tissues or physiological circumstances. Serine proteases encode protein cleaving enzymes that are involved in digestion, tissue remodeling, blood clotting etc., and many of the kallikrein genes are synthesized as precursor proteins that must be activated by cleavage of the propeptide. The predicted trypsin-like cleavage specificity of KLK-L2 makes it a  
25 candidate activator of other kallikreins or it may be involved in a "cascade" of enzymatic reactions similar to those found in fibrinolysis and blood clotting (36).

In conclusion, a new member of the human kallikrein gene family, KLK-L2 was characterized. This gene is hormonally regulated and it is mostly expressed in the brain, mammary gland and testis. KLK-L2 may be useful as a tumor marker.

#### Example 4

##### Materials and methods

##### Strategy for new gene discovery

Sequencing data of approximately 300 kb, around chromosome 19q13.3-q13.4, was obtained from the web site of the Lawrence Livermore National Laboratory (LLNL) (<http://www-bio.llnl.gov/genome/genome.html>). Different computer programs were used for putative new gene prediction, as previously  
35 described.

##### RT-PCR for KLK-L3 cDNA

Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA.

cDNA was prepared as described below and used for PCR amplification. A primer set (L3-F1 and L3-R1) was used to identify the presence of the gene in tissues, and the reverse primer (L3-R1) was used with another primer (L3-F2) to amplify and clone the full cDNA of the gene. These primer sequences are shown in Table 13 (SEQ. ID. Nos. 9-12, 24-26). Tissue cDNAs were amplified at various dilutions.

#### 5 Reverse transcriptase polymerase chain reaction.

2 µg of total RNA was reverse-transcribed into first strand cDNA using the Superscript<sup>TM</sup> preamplification system (Gibco BRL, Gaithersburg, MD). The final volume was 20 µl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequence, two gene-specific primers (L3-F1 and L3-R1) were designed (Table 13, SEQ. ID. Nos. 9-12, 24-26) and PCR was carried out in a reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 minutes, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute, and a final extension at 63°C for 10 minutes. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

#### Breast cancer cell line and hormonal stimulation experiments

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of 10<sup>-8</sup> M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for total RNA extraction by the Trizol method (Gibco-BRL). cDNA was prepared and amplified as described above. Control genes (PSA, pS2, and actin) were amplified as previously described herein.

#### Cloning and sequencing of the PCR products.

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

#### Identification of positive PAC and BAC genomic clones from human genomic DNA libraries

The PCR product generated with primer set Z1S and Z1AS (Table 14, SEQ.ID.NOS. 27-42), was purified and then labeled with <sup>32</sup>P by the random primer method (Sambrook, supra) and used as a probe to screen a human genomic DNA BAC library, spotted in duplicate on nylon membranes, for identification of positive clones. The filters were hybridized in 15% formamide, 500 mM Na<sub>2</sub>HPO<sub>4</sub>, 7% SDS, 1% BSA (w/v) at 65°C overnight, then washed sequentially with 2X SSC, 1X SSC, 0.2X SSC, containing 0.1% SDS

at 65°C, and then exposed to X-ray film as described (Sambrook, supra). Positive clones were obtained, plated on selective LB medium, and then a single colony was transferred into LB broth for overnight cultures. A PAC clone positive for NES1 was identified by a similar methodology as described elsewhere (14). PAC and BAC libraries were constructed by de Jong and associates (42). Purification of BAC and PAC DNA was done by a rapid alkaline lysis miniprep method, which is a modification of the standard Qiagen-Tip method. Positive clones were further confirmed by Southern blot analysis as described (Sambrook, supra).

#### 5' Rapid amplification of cDNA ends (5' RACE)

According to the EST sequences and the computer-predicted structure of the KLK-L3 gene, two gene specific primers were designed. Two rounds of RACE reactions (nested PCR) were performed with 5µl Marathon Ready™ cDNA of human testis (Clontech) as a template. The reaction mix and PCR conditions were selected according to the manufacturer's recommendations. Positive bands were gel-purified using Qiagen Gel Purification kits according to manufacturer's recommendations.

#### Gene-specific amplification of other genes from genomic DNA

According to the published sequence of prostatic specific antigen (PSA), human renal kallikrein (KLK1), human glandular kallikrein (KLK2), normal epithelial cell-specific Igene (NES1), KLK-L1, KLK-L2 and zyme genes, gene-specific primers were designed for each of these genes (Table 14) and developed polymerase chain reaction (PCR)-based amplification protocols which allowed us to generate specific PCR products with genomic DNA as a template. The PCR reactions were carried out as described above but by using an annealing/extension temperature of 65°C.

#### Structure analysis studies.

Multiple alignment was performed using the clustal X software package available at: <ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx1.64b.msw.exe> and the multiple alignment program available from the Baylor College of Medicine (BCM) search launcher (<http://kiwi.imgen.bcm.tmc.edu:8808/search-launcher/launcher/html>). Phylogenetic studies were performed using the Phylip software package available from: (<http://evolution.genetics.washington.edu/phylip/getme.html>). Distance matrix analysis was performed using the "Neighbor-Joining/UPGMA" program and parsimony analysis was done using the "Promoters" program. Hydrophobicity study was performed using the BCM search launcher programs (<http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html>). Signal peptide was predicted using the SignalP WWW server (<http://www.cbs.dtu.dk/services/signal>). Protein structure analysis was performed by SAPS (structural analysis of protein sequence) program (<http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html>).

#### Results:

#### Construction of a contiguous map of the human kallikrein locus on chromosome 19q13.3-q13.4

Sequence information around the human chromosome 19q13.3-q13.4 locus (the proposed kallikrein locus) is available at the Lawrence Livermore National Laboratory web site. Sequences of approximately 300 kb in length were obtained. These sequences were in the form of contigs of different lengths. A restriction analysis study of the contigs was performed using various computer programs. With

the aid of the EcoRI restriction map of this area which is also available at the LLNL web site, the relative positions of these contigs was defined in relation to each other. Some contigs were overlapping, enabling construction of a contiguous segment; however, three gaps were present. <BLAST> analysis of these segments against the GenBank database (37) enabled the precise location of two classical kallikreins, namely PSA and KLK2 to be defined. Other newly discovered serine proteases were localized which are homologous with the kallikrein genes, namely protease M/zyme/neurosin (10, 11, 12), human stratum corneum chymotryptic enzyme ( HSSCE) (55), neuropsin (28), normal epithelial cell-specific 1 gene ( NES1) (13), trypsin-like serine protease ( TLSP) (GenBank accession # AF164623), KLK-L1 (SEQ.ID.NO. 1) and KLK-L2 (SEQ.ID.NO. 13). The gaps in the 300 kb genomic sequence were partially

- (a) The margins of the first gap were found to contain the 5' and 3' ends of the KLK2 gene; this gap was filled with the genomic structure of the KLK2 gene (GenBank Accession # M18157).
- (b) The margins of the third gap (gaps are numbered from centromere to telomere) were found to have the 3' and 5' ends of the zyme gene mRNA sequence; thus, a radiolabeled probe specific for the zyme gene was used to screen a human BAC library and two positive clones were obtained. Restriction analysis was performed, followed by Southern blotting and a fragment containing the zyme gene was obtained and sequenced, thus filling this gap.
- (c) The second gap (between KLK-L1 and KLK-L2 genes) still exists and the EcoRI restriction map of this area was used to approximately define its length (Figure 14).

Further support for the relative locations of these genes was obtained by performing PCR reactions with gene-specific primers to screen genomic DNA clones. The most centromeric group of genes (PSA, KLK2, KLK-L1, KLK-L2 and zyme ) were found to be clustered in one genomic BAC clone, and the next group (HSCCE, neuropsin, KLK-L3 and NES1) were found to be clustered together in another clone, as expected from the data of Figure 14.

#### Cloning of the KLK-L3 gene

A putative new gene, formed of three exons, was predicted by computer analysis of the genomic sequence. The predicted exons were subjected to sequence homology search against the human EST database (dbEST) and revealed an EST clone (GenBank accession # AA583908) which exhibited 99% homology with the putative gene. This EST was obtained, purified and sequenced and the sequence was aligned by BLAST software (37) against the genomic area that contains the putative gene. An additional exon, downstream of the predicted structure, was identified. The 3' end of the gene was verified by: (a) The presence of the serine residue (S) of the catalytic triad in a well-conserved region. This highly conserved motif (GDSGGP) always occurs at the beginning of the last exon in all known kallikreins. (b) The presence of a stop codon that is in frame with the predicted amino acid sequence. (c) The presence of a 19-poly A stretch at the end of the EST that was not found in the genomic sequence.

To verify the accuracy of the cDNA sequence of the gene, PCR reactions were performed using gene-specific primers for the first and last exons of the predicted structure of the gene (L3-F2 and L3-R1) with cDNA isolated from different human tissues as putative templates. A positive band of the expected

size was isolated from testis cDNA and fully sequenced. Its sequence was aligned by BLAST against the genomic sequence to unequivocally define the exon/intron boundaries. For further characterization of the 5' end of the gene, 5'RACE reaction was performed using Marathon Ready cDNA from testis as a template. This allowed identification of an additional exon that contains the start codon and 5' untranslated region.

- 5 The full sequence of the gene is shown in SEQ. ID. NO. 21 (GenBank Accession # AF135026) and the amino acid sequences of KLK-L3 proteins are shown in SEQ. ID. Nos. 22 and 23.

**Structural characterization of the KLK-L3 gene:**

- As shown in Figure 15, the KLK-L3 gene is formed of 5 coding exons and 4 intervening introns, although, as with other kallikreins, the presence of further upstream untranslated exon(s) could not be ruled out (14, 28). All of the exon /intron splice sites conform to the consensus sequence for eukaryotic splice sites (32). The gene further follows strictly the common structural features of the human kallikrein multigene family, as described below.

- The predicted protein-coding region of the gene is formed of 753 bp, encoding a deduced amino acid polypeptide with a predicted molecular weight of 27.5 kDa. A potential translation initiation codon is found at position 28 of the predicted first exon (numbers of nucleotides refer to SEQ. ID. NO. 21 or GenBank Accession # AF135026. This codon does not match well with the consensus Kozak sequence (33), however, it has a purine at position (-3) which occurs in 97% of vertebrate mRNAs (43), and it is almost identical to the sequence of the zyme gene flanking the start codon. It should also be noted that most kallikreins do not have the consensus G nucleotide in position (+4).

- 20 Nucleotides 6803-6808 (AGTAAA) closely resemble a consensus polyadenylation signal (44) and are followed by a stretch of 19 poly A nucleotides not found in genomic DNA, after a space of 14 nucleotides. No other potential polyadenylation signals were discernable in the 3' untranslated region, suggesting that the above motif is indeed the polyadenylation signal. The same polyadenylation signal motif was predicted for the KLK1 and KLK2 genes.

- 25 Although the KLK-L3 protein sequence is unique, comparative analysis revealed that it is highly homologous to other members of the kallikrein multigene family. KLK-L3 shows 40% protein identity with the TLSP gene product and 38% and 33% identity with the KLK-L2 and KLK1 proteins, respectively. Hydrophobicity analysis revealed that the amino-terminal region is quite hydrophobic (Figure 16), consistent with the possibility that this region may harbor a signal sequence, analogous to other serine proteases. Computer analysis of the amino acid sequence of KLK-L3 predicted a cleavage site between amino acids 19 and 20 (GWA-DT). Sequence alignment (Figure 17) also revealed a potential cleavage site (Arg<sup>23</sup>), at a site homologous to other serine proteases (lysine (K) or arginine (R) is present in most cases). Several evenly distributed hydrophobic regions throughout the KLK-L3 polypeptide are consistent with a globular protein, similar to other kallikreins and serine proteases. The dotted region in Figure 17 indicates an 11-amino acid loop characteristic of the classical kallikreins (PSA, KLK1, and KLK2) but not found in KLK-L3 or other members of the kallikrein multi-gene family (11, 41).

35 Twenty nine "invariant" amino acids surrounding the active site of serine proteases have been described. Of these, twenty-six are conserved in KLK-L3. One of the unconserved amino acids (Ser<sup>168</sup>

instead of Pro) is also found in prostase, KLK-L2 and enamel matrix serine proteinase (EMSP1) genes. The second (Leu<sup>58</sup> instead of Val) is also found in TLSP and KLK-L2 genes, and the third is Ala<sup>26</sup> instead of Gly. According to protein evolution studies, each of these changed amino acids represents a conserved evolutionary change to a protein of the same group (45). Twelve cysteine residues are present in the putative mature KLK-L3 protein, ten of them are conserved in all the serine proteases that are aligned in Figure 17, and would be expected to form disulphide bridges. The other two (C<sup>136</sup> and C<sup>235</sup>) are not found in PSA, KLK1, KLK2 or trypsinogen; however, they are found in similar positions in prostase, HSCCE, zyme neuropsin, and TLSP genes and are expected to form an additional disulphide bond.

To predict the phylogenetic relatedness of the KLK-L3 gene with other serine proteases, the amino acid sequences of the kallikrein genes were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-joining/UPGMA method (Figure 18). Phylogenetic analysis separated the classical kallikreins (KLK1, KLK2, and PSA) and grouped KLK-L3 with TLSP, neuropsin, zyme, HSCCE and prostase/KLK-L1, consistent with previously published studies (11, 41).

#### Tissue expression and hormonal regulation of the KLK-L3 gene

As shown in Figure 19, the KLK-L3 gene is primarily expressed in thymus, testis, spinal cord, cerebellum, trachea, mammary gland, prostate, brain, salivary gland, ovary and skin (the latter two tissues are not shown in the figure). Lower levels of expression are seen in fetal brain, stomach, lung, thyroid, placenta, liver, small intestine, and bone marrow. No expression was seen in uterus, heart, fetal liver, adrenal gland, colon, spleen, skeletal muscle, pancreas, and kidney. In order to verify the RT-PCR specificity, representative PCR products were cloned and sequenced. Figure 20 shows that KLK-L3 gene is regulated by steroid hormones in the human breast cancer cell line BT-474.

#### DISCUSSION

A human kallikrein gene locus has been defined, and the first detailed map describing the relative positions of the kallikreins and other kallikrein-like genes has been constructed (Figure 14). This map is consistent with previous reports on the localization of the classical kallikreins and the approximate mapping of some new kallikreins by radiation hybrid and FISH techniques (9, 14, 67). It should be noted, however, that the lengths of certain segments of this map (as depicted in Figure 14) are dependent on the EcoRI restriction map of the area and are measured in terms of approximate kb units. Also, the measure of intervals between genes may change slightly in the future, since some kallikreins may have extra 5' exon(s) that have not as yet been identified. Kallikreins with verified 5'-untranslated exons include NES1 (14), zyme, and neuropsin (35). This map is also directional; it indicates that PSA and KLK2 genes are transcribed in the same direction (centromere to telomere) and that the rest of the kallikrein-like genes are transcribed in the reverse direction (Figure 14).

An early report indicated that KLK1 is located approximately 31 kb centromeric to PSA (9). The map described extends only 24 kb centromeric to PSA, and for this reason, KLK1 was not precisely localized. Thus, the exact location of the KLK1 gene is still to be defined from linear chromosome 19 sequencing data. The possibility still exists that this locus is extended further, and that other kallikrein-like

genes may be located upstream of PSA or downstream from TLSP.

Traditionally, kallikreins are characterized by their ability to liberate lysyl-bradykinin (kallidin) from kininogen (2). In humans, only KLK1 meets this "functional" definition. KLK2 and KLK3 are assigned to the same family based on the strong structural similarities of the genes and proteins and the close localization of these genes on the same chromosomal region (20). More recently, a new structural concept has emerged to describe kallikreins. Richards and co-workers introduced the concept of a "kallikrein multigene family" in mice, to refer to these genes (20, 21). This definition is not based much on the specific enzymatic function of the gene product, but more on its sequence homology and its close linkage on mouse chromosome 7.

Irwin et al. (27) proposed that the serine protease genes can be classified into five different groups according to intron position as discussed above. The results indicate the presence of some more common structural features that are found in all kallikreins (including the newly identified KLK-L3 gene): (1) All genes are formed of 5 coding exons and 4 intervening introns (with the possibility that some genes may have extra 5' untranslated exon(s) (24, 31, 35) (Figure 21). (2) The exon lengths are usually comparable. (3) The intron phases are always conserved (I-II-I-0) (see Figure 21 for description of intron phases). (4) These genes are clustered in the same chromosomal region, apparently without any intervening non kallikrein-like genes (Figure 14). Thus, all the recently identified serine proteases that are present in this region (zyme, HSCCE, neuropsin, NES1, prostase/KLK-L1, KLK-L2 and TLSP), together with the newly identified kallikrein-like gene (KLK-L3), could be considered members of the expanded human kallikrein multigene family.

The chromosomal band 19q13 is nonrandomly rearranged in a variety of human solid tumors including ovarian cancers (46), and the currently available data indicate that the kallikrein gene locus is related to many malignancies. At least three kallikrein genes (PSA, zyme and NES1) are down regulated in breast cancer (10, 13, 47, 48), and NES1 appears to be a novel tumor suppressor gene (29). Furthermore, PSA exhibits potent antiangiogenic activity (49). It is possible that some of these kallikreins are involved in a cascade pathway, similar to the coagulation or apoptotic process, whereby pro-forms of proteolytic enzymes are activated and then act upon downstream substrates. Such activity was found for the KLK2 gene product which acts upon and activates pro PSA (50, 51).

The expanded human kallikrein gene family has similar number of members as the rodent family of genes. Some new compelling data have raised the possibility that at least some of these genes behave as tumor suppressors (29), as negative regulators of cell growth (52), as antiangiogenic (49) and apoptotic (53) molecules. The paramount diagnostic value of some members is also well-established (24, 54). For these reasons, it is important to check all members of this family of genes as potential diagnostic or prognostic markers or as candidate therapeutic targets.

The newly identified KLK-L3 gene is expressed in many tissues, including skin, thymus, central nervous system, breast, prostate, and testis. The wide range of tissue expression of KLK-L3 should not be surprising since, by using the more sensitive RT-PCR technique, many kallikrein genes were found to be expressed in a wide variety of tissues. For example, PSA, KLK2, prostase/KLK-L1, and KLK-L2 are now

known to be expressed in breast and many other tissues (30, 54).

Like many other kallikreins, KLK-L3 is regulated by steroid hormones but in a more complex fashion than PSA and KLK2 which are up-regulated by androgens and progestins (71). In the breast carcinoma cell line studied, KLK-L3 appears to be up-regulated by progestins > estrogens > androgens (Figure 20).

### Example 5

#### Materials and Methods

##### DNA sequence on chromosome 19 and prediction of new genes

Sequencing data of approximately 300Kb of nucleotides, around chromosome 19q13.3-q13.4, was obtained from the web site of the Lawrence Livermore National Laboratory (LLNL) ([http://www-http://www-bio.llnl.gov/genome/genome.html](http://www.http://www-bio.llnl.gov/genome/genome.html)) and an almost contiguous stretch of genomic sequences was constructed. A number of computer programs were used to predict the presence of putative new genes in this genomic area.

##### Expressed sequence tag (EST) searching

The predicted exons of the putative new gene were subjected to homology search using the BLASTN algorithm (37) on the National Center for Biotechnology Information web server ([http://www-ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)) against the human EST database (dbEST). Clones with > 95% homology were obtained from the I.M.A.G.E. consortium (38) through Research Genetics Inc, Huntsville, AL. The clones were propagated, purified and sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

##### Rapid amplification of cDNA ends (3' RACE)

According to the EST sequence data and the predicted structure of the gene, two gene-specific primers were designed and two rounds of RACE reactions (nested PCR) were performed with 5µl Marathon Ready™ cDNA of human testis (Clontech, Palo Alto, CA, USA) as a template. The reaction mix and PCR conditions used were according to the manufacturer's recommendations.

##### Tissue expression

Total RNA isolated from 26 different human tissues was purchased from Clontech. cDNA was prepared as described below, and used for PCR reactions with different sets of primers (Table 15, SEQ.ID.NOs. 46-55, 9-12). Tissue cDNAs were amplified at various dilutions.

##### Breast cancer cell line and hormonal stimulation experiments

The breast cancer cell line BT-474 was purchased from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media, at a final concentration of  $10^{-8}$  M. Cells stimulated with 100% ethanol were included as controls.



The cells were cultured for 24 hours, then harvested for mRNA extraction.

#### Reverse transcriptase polymerase chain reaction

Total RNA was extracted from the breast cancer tissues and cell lines using Trizol™ reagent (Gibco BRL) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2 µg of total RNA was reverse-transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20 µl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (L4-F1 and L4-R1, see Table 15, SEQ.ID.NOS. 46 and 47) and PCR was carried out in a reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling conditions were 94°C for 9 minutes to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute and a final extension at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

#### Normal and malignant breast tissues

Normal breast tissues were obtained from women undergoing reduction mammoplasties. Breast tumor tissues were obtained from female patients at participating hospitals of the Ontario Provincial Steroid Hormone Receptor Program. The normal and tumor tissues were immediately frozen in liquid nitrogen after surgical resection and stored in this manner until extracted. The tissues were pulverized with a hammer at dry ice temperature and RNA was extracted as described above, using Trizol reagent.

#### Structure analysis

Multiple alignment was performed using the Clustal X software package available at: [ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx/\[clustalx1.64b.msw.exe\]](ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx/[clustalx1.64b.msw.exe]) and the multiple alignment program available from the Baylor College of Medicine (BCM), Houston, TX, USA [kwi.imgen.bcm.tmc.edu:8808/search-launcher/launcher/html]. Phylogenetic studies were performed using the Phylip software package available at: <http://evolution.genetics.washington.edu/phylip/getme.html>. Distance matrix analysis was performed using the "Neighbor-Joining/UPGMA" program and parsimony analysis was done using the "Protpars" program. Hydrophobicity study was performed using the BCM search launcher programs [<http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html>]. Signal peptide was predicted using the "SignalP" server [<http://www.cbs.dtu.dk/services/signal>]. Protein structure analysis was performed by the "SAPS" (structural analysis of protein sequence) program [<http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html>].

#### Results

### Cloning of the KLK-L4 gene

Computer analysis of the genomic sequence around chromosome 19 q13.3-q13.4 predicted a putative new gene formed of at least 3 exons. To experimentally verify the existence of this gene, the putative exons were subjected to sequence homology search against the human expressed sequence tag (EST) database (dbEST), and four EST clones with > 97% homology were identified (Table 16). All ESTs were cloned from testicular tissue. These clones were obtained and inserts were sequenced from both directions. Sequences were then compared with the computer-predicted structure and final selection of the intron/exon splice sites was made according to the EST sequences.

As shown in Figure 22, three ESTs match almost perfectly with the predicted 3 exons (exons 3, 4, 5) of the gene and one EST matches perfectly with predicted exons 3 and 5. However, each of the ESTs extends further upstream with different exonic patterns, suggesting the presence of different splice variants. Attempts to translate these clone sequences demonstrated the presence, in some ESTs, of interrupting stop codons in all three possible reading frames. A homology search of the three common exons against the GenBank database revealed a cDNA sequence from the German Human Genome Project. This clone has an identical exon 2 as the long form of KLK-L4 gene [this form will be described below] but has an extended exon 3 that ends with a stop codon (Figure 22). This clone was isolated from uterine tissue and is translated by software into a truncated protein product of 196 amino acids which is followed by a 3' untranslated region [GenBank accession # AL050220].

Screening of cDNAs from 26 different tissues by RT-PCR, using gene-specific primers for exons 3 and 5 [L4-F1 and L4-R1] (Table 15 & Figure 22) revealed that this gene is expressed in many tissues. Four tissues that show the highest level of expression [salivary gland, mammary gland, prostate, and testis] (Figure 23) and uterus [the EST clone AL050220 was isolated from this tissue] were selected for identification of the full structure of the gene. Different PCR reactions were performed using one reverse primer [L4-R1] together with each of the forward primers located in upstream exons that were found in the different EST clones [primers L4-B, L4-D, L4-E] (Table 15 & Figure 22). The PCR reactions were performed under different experimental conditions, using the EST clones as positive controls, and the PCR products were sequenced. None of these forms were found in any of the tissues, except in testis where all three forms were found.

By RT-PCR of the KLK-L4 gene using primers L4-R1 and L4-F1, it was found that the gene is expressed in a wide variety of tissues (Figure 23). In order to obtain the structural forms that exist in these tissues, a homology study was performed. Aligning the predicted polypeptide of the KLK-L4 gene with all other kallikreins and kallikrein-like genes, suggested, by homology, that at least two more exons should be present upstream of the predicted three exons. The genomic fragment upstream of the third exon was subjected to further computer analysis for gene prediction, and exon 2 was identified based on: a) a consensus exon/intron splice site b) preservation intron phase II after this exon, in agreement to intron phases of all other known kallikreins c) presence of the histidine residue of the catalytic triad (H<sup>76</sup>) surrounded by a well-conserved peptide motif [see below] just before the end of this exon d) comparable exon length to other kallikrein genes. A potential first exon was also predicted from the upstream genomic

sequence, based on the preserved intron phase (phase I), and the existence of an in-frame start codon that is located at a comparable distance [in relation to other kallikreins] from the end of this exon. To verify this predicted structure, a PCR reaction was performed using one reverse primer (L4-R1) together with another forward primer that is located in the predicted first exon (primer L4-X1) (Table 15 & Figure 22).

Two main PCR bands were obtained from the tissues examined; the expected 819 bp band (predominant) and an additional minor band of about 650 bp (Figure 24). Cloning and sequencing of these two bands revealed that the gene exists in two main forms in these tissues; the long form [SEQ. ID. No. 43 or GenBank Accession No. AF135024] and another form [referred to as the short KLK-L4 variant] that utilizes an upstream alternative splice donor site, located inside exon 3, thus creating an mRNA product that is 214 bp shorter. This alternative splice site causes frame-shifting of the coding region that will generate a predicted stop codon at the beginning of exon 4, giving rise to a truncated protein product that does not contain the serine residue of the catalytic triad (Figures 24 and 25).

Aligning the long KLK-L4 form with the ESTs (Figure 22) demonstrated that all ESTs utilize a different splice donor site located 80 bp downstream from the end of exon 3. These additional 80 bp contain an in-frame stop codon at nucleotide position 5505 which will lead to the formation of a shorter polypeptide product. They also utilize an alternative polyadenylation signal located at position 8706 [numbers refer to SEQ. ID. No. 43 or GenBank Accession No. AF135024]. The clone from the German Genome Project utilizes another splice donor site that is located further downstream, inside intron 3, and ends up with a poly A tail without having a fourth or fifth exon. The same stop codon (position 5505) will be in-frame, and therefore, a truncated protein product is predicted to be formed (Figure 22).

In order to obtain the 3' end of the gene, a 3'RACE reaction was performed, and an additional 375 bp fragment of 3' untranslated region, downstream from PCR primer L4-R1, was obtained. This fragment was further confirmed to be present in all tissues tested, by performing a PCR reaction using primers L4-F1 and L4-R3 (Table 15 & Figure 22). This fragment ends with a putative polyadenylation signal variant (TATAAA).

#### **Structural characterization of the KLK-L4 gene and its protein product**

The long form of the KLK-L4 gene is presented in Figure 25 (SEQ.ID.NO. 43). KLK-L4 is formed of five coding exons and four intervening introns, spanning an area of 8,905 bp of genomic sequence on chromosome 19q13.3-q13.4. The lengths of the coding regions are 52, 187, 269, 137 and 189 bp, respectively. The predicted protein coding region of the gene is formed of 831 bp, encoding a deduced 277-amino acid protein with a predicted molecular mass of 30.6 kDa (Figure 25). The intron/exon splice sites (mGT....AGm, where m is any base) and their flanking sequences are in agreement with the consensus splice site sequence. A potential translation initiation codon is present at position 45 of the predicted first exon [numbers refer to SEQ. ID. No. 43]. The cDNA extends at least 382 bp further downstream from the stop codon and a putative polyadenylation signal (TATAAA) is present at the end of this region (Figure 25).

Hydrophobicity analysis revealed that the amino-terminal region is quite hydrophobic (Figure 26), consistent with the possibility that this region may harbor a signal sequence, analogous to other serine

proteases. Figure 26 also shows the presence of several evenly distributed hydrophobic regions throughout the KLK-L4 polypeptide, which are consistent with a globular protein, similar to other serine proteases (13). Computer analysis of the amino acid sequence of KLK-L4 predicted a cleavage site between amino acids 20 and 21 (GVS-QE). Sequence homology with other serine proteases (Figure 27) predicted another potential cleavage site (Lys25) in close proximity. Most other kallikreins are activated by cleavage after arginine or lysine. Thus, the protein product is very likely to be a secreted protein. The dotted region in Figure 27 indicates an 11-amino acid loop characteristic of the classical kallikreins (PSA, KLK1, and KLK2) which is not found in KLK-L4 or other members of the kallikrein multi-gene family (11,13, 35).

Amino acid sequences for KLK-L4 proteins are shown in SEQ.ID.NO. 44 and 45.

Sequence analysis of eukaryotic serine proteases indicates the presence of twenty nine invariant amino acids (39). Twenty eight of them are conserved in the KLK-L4 protein and the remaining amino acid (Q182 instead of P) is not conserved among all other kallikreins (Figure 27). Ten cysteine residues are present in the putative mature KLK-L4 protein. These are conserved in all the serine proteases that are aligned in Figure 27, and would be expected to form disulphide bridges. The presence of aspartate (D) in position 239 suggests that KLK-L4 will possess a trypsin-like cleavage pattern, similarly to most of the other kallikreins [e.g., KLK1, KLK2, TLSP, neuropsin, zyme, prostase, and EMSP] but different from PSA which has a serine (S) residue in the corresponding position, and is known to have chymotrypsin like activity (Figure 27) (2,40).

#### Mapping and chromosomal localization of the KLK-L4 gene

Alignment of the KLK-L4 gene and the sequences of other known kallikrein genes within the 300 Kb area of interest [the human kallikrein gene family locus], enabled precise localization of all known genes and to determine the direction of transcription, as shown by the arrows in Figure 28. The PSA gene lies between KLK1 and KLK2 genes and is separated by 13, 319 base pairs (bp) from KLK2, and both genes are transcribed in the same direction (centromere to telomere). All other kallikrein-like genes are transcribed in the opposite direction. KLK-L4 is 13 kb centromeric from KLK-L6 [SEQ.ID.NO. 65], and 21 kb more telomeric to KLK-L5 [SEQ. ID. NO. 56].

#### Homology with the kallikrein multi-gene family

Alignment of the amino acid sequence of the KLK-L4 protein (long form) against the GenBank database and the known kallikreins, using the BLAST algorithm (37), indicated that KLK-L4 has 51% amino acid sequence identity with the TLSP and zyme genes, 49% identity with KLK-L2 and 47% and 45% identity with PSA and KLK2 genes, respectively. Multiple alignment study shows that the typical catalytic triad of serine proteases is conserved in the KLK-L4 gene (H<sup>108</sup>, D<sup>153</sup>, and S<sup>245</sup>) and, as is the case with all other kallikreins, a well conserved peptide motif is found around the amino acid residues of the catalytic triad [i.e. histidine (WLTAHHC), serine (GDSGGP), and aspartate (DLMLI)] (Figure 27) (1, 11, 13, 35).

In addition, several other residues were found to be fully or partially conserved among the human kallikrein gene family, as further shown in Figure 27. To predict the phylogenetic relatedness of the KLK-L4 gene with other serine proteases, the amino acid sequences of the kallikrein genes were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-

joining/UPGMA method (Figure 29). Phylogenetic analysis separated the classical kallikreins (KLK1, KLK2, and PSA) and grouped KLK-L4 with zyme, TLSP, KLK-L3, neuropsin, and NES1 genes, consistent with previously published studies (41) and indicating that this group of genes probably arose from a common ancestral gene by duplication.

#### 5 Tissue expression and hormonal regulation of the KLK-L4 gene

As shown in Figure 23, the KLK-L4 gene is primarily expressed in mammary gland, prostate, salivary gland and testis, but, as is the case with other kallikreins, lower levels of expression are found in many other tissues. In order to verify the RT-PCR specificity, the PCR products were cloned and sequenced.

- 10 A steroid hormone receptor-positive breast cancer cell line (BT-474) was used as a model, to verify whether the KLK-L4 gene is under steroid hormone regulation. PSA was used as a control gene, known to be up-regulated by androgens and progestins and pS2 as an estrogen up-regulated control gene in the same cell line. Preliminary results indicate that KLK-L4 is up-regulated by progestins and androgens and to a lower extent by estrogens (Figure 30).

#### 15 Expression of KLK-L4 in breast cancer tissues and cell lines

- To characterize the extent and frequency of expression of the KLK-L4 gene in breast tumors, cDNA derived from 3 normal and 19 malignant breast tissues and 3 breast cancer cell lines was used. The data were interpreted by comparison of band intensities. Out of the 19 tumors, KLK-L4 gene expression was undetectable in 7, lower than normal tissues in 9, comparable to the normal tissues in 1, and higher than  
20 normal tissues in 2 tumors. Without hormonal stimulation, the BT-474 and T-47D cell lines had no detectable KLK-L4 mRNA, while the MCF-7 cell line was positive. These preliminary results suggest that this gene is down-regulated in the majority (16/19) of breast tumors.

#### Discussion

- The established kallikreins (KLK1, KLK2, and PSA), trypsinogen and chymotrypsinogen belong  
25 to a group that has: (1) an intron just downstream from the codon for the active site histidine residue, (2) a second intron downstream from the exon containing the codon for the active site aspartic acid residue, and (3) a third intron just upstream from the exon containing the codon for the active site serine residue. Figure 31 shows that KLK-L4 meets the above mentioned criteria; moreover, is located in close proximity to other kallikrein genes on the chromosomal locus 19q13.3-q13.4 (Figure 28).

- 30 The preliminary findings, supporting that the KLK-L4 gene may be down-regulated in a subset of breast cancers, is not surprising. There is now growing evidence that many of the kallikreins and kallikrein-like genes that are clustered in the same chromosomal region (Figure 28) are related to malignancy. PSA is the best marker for prostate cancer so far (24). A recent report provided evidence that PSA has antiangiogenic activity, and that this activity may be related to its function as a serine protease (49). This study suggested that other serine proteases, including new members of the kallikrein multigene family of enzymes, should also be evaluated for potential antiangiogenic activity (49). Recent reports suggest that hK2 (encoded by the KLK2 gene) could be another useful diagnostic marker for prostate cancer (57, 58). NES1 appears to be a tumor suppressor gene (29). The protease M gene was shown to

be differentially expressed in primary breast and ovarian tumors (10), and the human stratum corneum chymotryptic enzyme has been shown to be expressed at abnormally high levels in ovarian cancer (59). Another recently identified kallikrein-like gene, located close to KLK-L4 and tentatively named tumor-associated, differentially expressed gene-14 (TADG14) [an alternatively spliced form of neuropsin, see Figure 28] was found to be overexpressed in about 60% of ovarian cancer tissues (59). Also, prostate/KLK-L1, another newly discovered kallikrein-like gene, is speculated to be linked to prostate cancer (41). Thus, extensive new literature suggests multiple connections of many kallikrein genes to various forms of human cancer.

The removal of intervening RNA sequences (introns) from the pre-messenger RNA in eukaryotic nuclei is a major step in the regulation of gene expression (60). RNA splicing provides a mechanism whereby protein isoform diversity can be generated and the expression of particular proteins with specialized functions can be restricted to certain cell or tissue types during development (60). The sequence elements in the pre-mRNA at the 5' and 3' splice sites in metazoans have very loose consensus sequence; only the first and the last two bases (GT..AG) of the introns are highly conserved (Sambrook, supra). These sequences cannot be the sole determinants of splice site selection, since identical, but not ordinarily active, consensus sequences can be found within both exons and introns of many eukaryotic genes. Other protein factors and sequences downstream of the splice sites are also involved.

The existence of multiple splice forms is frequent among kallikreins. Distinct RNA species are transcribed from the PSA gene, in addition to the major 1.6-kb transcript (61). Several distinct PSA transcripts have been described by Reigman et al (7). Interestingly, one of these clones lacks the 3' untranslated region and the first 373 nucleotides of the open reading frame, and has an extended exon that contains a stop codon, a pattern that is comparable with some alternative forms of the KLK-L4 cDNA, as described here (Figure 22). Heuze et al., reported the cloning of a full-length cDNA corresponding to a 2.1 kb PSA mRNA. This form results from the alternative splicing of intron 4 and lacks the serine residue that is essential for catalytic activity (61). Also, Reigman et al reported the identification of two alternatively spliced forms of the human glandular kallikrein 2 (KLK2) gene (62). A novel transcript of the tissue kallikrein gene (KLK1) was also isolated from the colon (63). Interestingly, this transcript lacks the first two exons of the tissue kallikrein gene, but the last three exons were fully conserved, a pattern that is similar to the findings with some ESTs containing parts of the KLK-L4 gene (Figure 22). Neuropsin, a recently identified kallikrein-like gene, was found to have two alternatively spliced forms, in addition to the major form (59, 64). Here, the cloning of the KLK-L4 gene is described and the identification of a number of alternative mRNA forms. These forms may result from alternative splicing (Sambrook, supra), retained intronic segment (7), or from the utilization of an alternative transcription initiation site (63). Because the long form of KLK-L4 and the major alternative splice variant [short KLK-L4 variant] (Figure 24) have an identical 5' sequence required for translation, secretion and activation, it is possible to assume that both mRNAs encode for a secreted protein (61).

In order to investigate the relative predominance of the long KLK-L4 and related forms, cDNA from various tissues was amplified by PCR. Although, in general, it is difficult to use PCR for quantitative

comparisons between mRNA species, in this experiment, [mRNAs of comparable sizes, using one set of primers under identical conditions], such a comparison is reasonable (62). In all five normal tissues examined [breast, prostate, testis, salivary gland and uterus] the long form of KLK-L4 was the predominant, with minimal level of expression of the short form (Figure 24).

The presence of alternatively spliced forms may be related to malignancy. Recent literature suggests that distinct molecular forms of PSA could be expressed differently by malignant versus benign prostate epithelium (65). Aberrant PSA mRNA splicing in benign prostatic hyperplasia, as opposed to prostate cancer, has been described by Henttu et al (66). In addition, it has been recently postulated that different prostatic tissues potentially harboring occult cancer could account for the presence of various forms of PSA (65).

### Example 6

#### Materials and Methods

##### DNA sequence on chromosome 19

Sequencing data of approximately 300Kb of nucleotides on chromosome 19q13.3-q13.4 was obtained from the web site of the Lawrence Livermore National Laboratory (LLNL) (<http://www-bio.llnl.gov/genome/genome.html>). This sequence was in the form of 9 contigs of different lengths. Restriction enzyme analysis, long PCR strategies, followed by DNA sequencing, BAC and PAC library screening and end sequencing of selected clones, were used to construct a contiguous genomic region, representing the complete human kallikrein gene locus.

##### New gene identification

A number of computer programs were used to predict the presence of putative new genes within the contiguous genomic area of interest. The ability of these programs for predicting new genes was first examined by using the genomic sequences of the known kallikreins as testing parameters. The most reliable computer programs; GeneBuilder (gene prediction) (<http://125.itba.mi.cnr.it/~webgene/genebuilder.html>), GeneBuilder (exon prediction) (<http://125.itba.mi.cnr.it/~webgene/genebuilder.html>), Grail 2 (<http://combio.ornl.gov>), and GENEID-3 (<http://apollo.imim.es/geneid.html>) were selected for further use.

##### Expressed sequence tag (EST) searching

The predicted exons of the putative new gene were subjected to homology search using the BLASTN algorithm (37) on the National Center for Biotechnology Information web server (<http://www.ncbi.nlm.nih.gov/BLAST/>) against the human EST database (dbEST). A clone with > 95% homology was obtained from the I.M.A.G.E. consortium (38) through Research Genetics Inc, Huntsville, AL. This clone was propagated, purified and sequenced from both directions with an automated sequencer, using insert-flanking vector primers.

##### Rapid amplification of cDNA ends (RACE)

According to the EST sequence and the predicted structure of the gene, two sets of gene-specific primers were designed for 5' and 3' RACE reactions. Two rounds of RACE reactions (nested PCR) were performed for each type of RACE with 5µl Marathon Ready™ cDNA of human testis and prostate (Clontech, Palo Alto, CA, USA) as templates. The reaction mix and PCR conditions were selected

according to the manufacturer's recommendations. In brief, the initial denaturation was for 5 min at 94°C, followed by 94°C for 5 s and 72°C for 2 min, for 5 cycles; then, 94°C for 5 s and 70°C for 2 min, for 5 cycles; then, 94°C for 5 s and 65°C for 2 min for 30 cycles for the first reaction and 25 cycles for the nested PCR reaction.

## 5 Tissue expression

Total RNA isolated from 26 different human tissues was purchased from Clontech, Palo Alto, CA. cDNA was prepared as described below for the tissue culture experiments and used for PCR reactions. After aligning all known kallikrein genes, two primers (KLK-L5-R1 and KLK-L5-F1) (Table 17, SEQ.ID.NO.s. 61-64, 9-12, and Figure 32) were designed from areas with relatively low homology. Tissue cDNAs were amplified at various dilutions. Due to the high degree of homology between kallikreins, and to exclude non-specific amplification, PCR products were cloned and sequenced.

### Normal and malignant breast tissues

Normal breast tissues were obtained from women undergoing reduction mammoplasties. Breast tumor tissues were obtained from female patients at participating hospitals of the Ontario Provincial Steroid Hormone Receptor Program. The normal and tumor tissues were immediately frozen in liquid nitrogen after surgical resection and stored in this manner until extracted. The tissues were pulverized with a hammer under liquid nitrogen and RNA was extracted as described below, using Trizol reagent.

### Breast and prostate cancer cell lines and hormonal stimulation experiments

The breast cancer cell lines BT-474 and T-47D, and the LNCaP prostate cancer cell line were purchased from the American Type Culture Collection (ATCC), Rockville, MD. Cells were cultured in RPMI media (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (10%), antibiotics and antimycotics, in plastic flasks, to near confluency. The cells were then aliquoted into 24-well tissue culture plates and cultured to 50% confluency. 24 hours before the experiments, the culture media were changed into phenol red-free media containing 10% charcoal-stripped fetal bovine serum. For stimulation experiments, various steroid hormones dissolved in 100% ethanol were added into the culture media at a final concentration of  $10^{-8}$  M. Cells stimulated with 100% ethanol were included as controls. The cells were cultured for 24 hours, then harvested for mRNA extraction.

### Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cell lines or tissues using Trizol reagent (Gibco BRL) following the manufacturer's instructions. RNA concentration was determined spectrophotometrically. 2 µg of total RNA was reverse-transcribed into first strand cDNA using the Superscript™ preamplification system (Gibco BRL). The final volume was 20 µl. Based on the combined information obtained from the predicted genomic structure of the new gene and the EST sequences, two gene-specific primers were designed (KLK-L5-F1 and KLK-L5-R1) (Table 17) and PCR was carried out in a reaction mixture containing 1 µl of cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs (deoxynucleoside triphosphates), 150 ng of primers and 2.5 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA) on a Perkin-Elmer 9600 thermal cycler. The cycling



conditions were 94°C for 9 minutes to activate the Taq Gold DNA polymerase, followed by 43 cycles of 94°C for 30 s, 63°C for 1 minute and a final extension step at 63°C for 10 min. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. All primers for RT-PCR spanned at least 2 exons to avoid contamination by genomic DNA.

To verify the identity of the PCR products, they were cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The inserts were sequenced from both directions using vector-specific primers, with an automated DNA sequencer.

#### Structure analysis

Multiple alignment was performed using the Clustal X software package available at: <ftp://ftp.ebi.ac.uk/pub/software/dos/clustalw/clustalx1.64b.msw.exe> and the multiple alignment program available from the Baylor College of Medicine (BCM), Houston, TX, USA ([kiwi.imgen.bcm.tmc.edu:8808/search-launcher/launcher/html](http://kiwi.imgen.bcm.tmc.edu:8808/search-launcher/launcher/html)). Phylogenetic studies were performed using the PhyIip software package available at: <http://evolution.genetics.washington.edu/phyIip/getme.html>. Distance matrix analysis was performed using the "Neighbor-Joining/UPGMA" program and parsimony analysis was done using the "Protpars" program. Hydrophobicity study was performed using the BCM search launcher programs (<http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html>). Signal peptide was predicted using the "SignalP" server (<http://www.cbs.dtu.dk/services/signal>). Protein structure analysis was performed by "SAPS" (structural analysis of protein sequence) program (<http://dot.imgen.bcm.tmc.edu:9331/seq-search/struc-predict.html>).

## RESULTS

### Identification of the KLK-L5 gene

Computer analysis of the genomic area of interest (300Kb around chromosome 19q13.3-q13.4) predicted a putative gene comprised of at least three exons. Screening of the human expressed sequence tag (EST) database revealed an EST clone (GenBank Accession #394679) with 99% homology with predicted exons. This clone was obtained, purified, and sequenced. The full-length sequence of the EST was compared with the genomic area containing the putative new gene and showed 100% homology with certain areas (exons), which were separated by introns. This alignment indicated that the new gene was comprised of 7 exons. Sequence homology comparisons and phylogenetic analysis revealed that this new gene is structurally similar to known kallikreins and other serine proteases (see below). Since four other new kallikrein-like genes were discovered in this area, this gene was named KLK-L5 (for kallikrein-like gene 5). Attempts to translate the coding region in all three possible reading frames indicated that only one reading frame will produce a full-length polypeptide chain without interrupting in-frame stop codons. Further support for the correctness of this reading frame was obtained by demonstrating that only this frame will preserve the three amino acid residues necessary for serine protease activity (catalytic triad) and the conserved motifs around them. An in-frame methionine start codon was found in the second exon. This start codon falls within a typical consensus Kozak sequence (CCACCATGG) (33). Thus, the gene will have at least one 5' untranslated exon, similarly to other kallikrein-like genes [e.g. zyme, the normal epithelial cell-specific 1 gene (NES1) (14), and neuropsin (35)]. 5' and 3' RACE reactions were performed in order to

obtain the 5' and 3' ends of the gene. No more sequence was obtained by 5' RACE. However, 3' RACE enabled identification of the 3' untranslated region of the gene. The additional sequence ends with a poly-A stretch that does not exist in the genomic structure, thus marking the 3' end of the gene and the start of the poly-A tail.

### Splice variants of the KLK-L5 gene

Homology analysis of the KLK-L5 gene with other kallikreins revealed the presence of an additional 3' exon, an observation that has not been reported for any other member of the kallikrein multigene family. Furthermore, two different PCR bands were obtained with the 3' RACE. Sequencing of these bands revealed that this gene has at least two splice forms at its 3' end; one form in which the last exon is a single continuous fragment, and another form in which the last exon is split into two exons, with an intervening intron. In order to identify the full structure of other possible splice variants of the gene, PCR was performed using two primers (L5-F2 and L5-R2) (Table 17 and Figure 32). cDNA from 26 different tissues were used as templates and the reaction was performed under different experimental conditions (annealing temperature,  $MgCl_2$  concentration). Three distinct bands were observed in many tissues. These bands were excised, gel-purified, and sequenced. As shown in Figure 32, the KLK-L5 gene was found to have 3 molecular forms:

1) One form (referred to, from now on, as the "classical" form) represents a typical kallikrein-like serine protease with five coding exons and four intervening introns (Figure 32). As is the case with some other kallikreins, a 5' untranslated exon is also present, and the possibility of further upstream untranslated exon(s) could not be excluded. Exons 1, 2 and 3 were present at the aforementioned EST. The start codon is present in the second exon (numbers refer to SEQ.ID.NO. 56 or GenBank Accession # AF135025). The stop codon is located in the sixth exon, followed by a 3' untranslated region, and a typical polyadenylation signal (AATAAA) is located 16 bp before the poly-A tail (Figure 33). This form of KLK-L5 spans a genomic length of 5,801 bp on chromosome 19q13.3-q13.4. The lengths of the coding regions of the exons are 37, 160, 260, 134, and 156 bp, respectively (Figures 33 and 34). The predicted protein-coding region is formed of 747 bp, encoding a deduced 248-amino acid protein with a predicted molecular mass of 26.7 kDa. The intron/exon splice sites (GT....AG) and their flanking sequences are in agreement with the consensus splice site sequence.

2) The second mRNA form, encoding the KLK-L5-related protein-1, is an alternatively spliced form in which the last exon is split into two separate exons with an additional intervening intron (Figure 32). This splitting of the last exon results in the utilization of another stop codon at position 9,478, thus creating a deduced 254-amino acid protein that is 6 amino acids longer than the "classical" KLK-L5 form and its carboxyterminal end is different in sequence by 19 amino acids (Figure 32). This variant has a predicted molecular mass of 27.1 kDa (for base numbering please see SEQ.ID.NO. 56 and GenBank Accession # AF135025).

3) The third mRNA form, encoding for KLK-L5-related protein-2, is similar to the classical form except that the fourth exon is missing (Figure 32). This leads to frame shifting of the coding region, and an earlier in-frame stop codon will be encountered at position 9,180. The protein-coding region of this form

consists of 336 bp, encoding for a predicted 111-amino acid protein with a molecular mass of 12 kDa. This protein will lack both the serine and aspartate residues characteristic of serine proteases.

Amino acid sequences for KLK-L5 proteins are shown in SEQ. ID. NOs. 57 to 60.

### Structural analysis of the classical KLK-L5 gene

Figure 35 shows a comparative hydrophobicity analysis of the KLK-L5 and the prostate-specific antigen (PSA) proteins. The amino terminal regions of both genes are quite hydrophobic, indicating that this region of KLK-L5 is possibly harboring a signal peptide analogous to PSA. Figure 35 also shows the presence of several evenly distributed hydrophobic regions throughout the KLK-L5 polypeptide, which are consistent with a globular protein, similar to other serine proteases (13). Figure 36 shows the alignment of KLK-L5 protein with another 10 members of the same gene family. The dotted region in Figure 36 indicates an 11-amino acid loop characteristic of the classical human kallikreins (PSA, hK1 and hK2) but not found in KLK-L5 protein or other members of the kallikrein multigene family (11, 13, 35). Sequence analysis of eukaryotic serine proteases indicates the presence of twenty nine invariant amino acids (39). Twenty eight of them are conserved in the KLK-L5 polypeptide and the remaining amino acid (S156 instead of P) is not conserved among all other kallikreins (Figure 36). Twelve cysteine residues are present in the putative mature KLK-L5 protein, ten of them are conserved in all kallikreins, and the remaining two (C133, and C235) are present in most of the other kallikrein-like proteins but not in the classical kallikreins and they are expected to form an additional disulphide bridge (Figure 36).

The presence of aspartate (D) at position 194 suggests that KLK-L5 will possess a trypsin-like cleavage pattern, similarly to most of the other kallikreins (e.g., hK1, hK2, TLSP, neuropsin, zyme, prostate, and EMSP) but different from PSA, which has a serine (S) residue in the corresponding position, and is known to have chymotrypsin like activity (Figure 36) (54).

### Homology with other members of the kallikrein multigene family

Although the protein encoded by the KLK-L5 gene is unique, it has a high degree of homology with the other kallikrein-like genes. The KLK-L5 protein (classical form) has 48% amino acid sequence identity and 57% overall similarity with neuropsin, 46% identity with the normal epithelial cell-specific 1 gene product (NES1) and 38% identity with both PSA and hK2 proteins. Multiple alignment shows that the typical catalytic triad of serine proteases is conserved in the KLK-L5 protein ( $H^{62}$ ,  $D^{106}$ , and  $S^{200}$ ) (Figures 33 and 36). In addition, a well-conserved peptide motif is found around the amino acid residues of the catalytic triad as is the case with other serine proteases [i.e., histidine (VLTA~~A~~HC), serine (GD~~S~~G~~G~~PP), and aspartate (DLRL~~L~~)] (11, 12) (Figure 36). Figure 36 also shows other amino acid residues that are completely conserved between kallikreins and kallikrein-like proteins. To predict the phylogenetic relatedness of the KLK-L5 protein with other serine proteases, the amino acid sequences of the kallikrein proteins were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-joining/UPGMA and Protpars parsimony methods. Figure 37 shows the phylogenetic analysis which separated the classical kallikreins (hK1, hK2, and PSA) and clustered KLK-L5 with NES1 and neuropsin proteins in a separate group away from other serine proteases, consistent with previously published studies (27, 41) and indicating that this group of genes probably arose from a common

ancestral gene, by gene duplication.

#### **Tissue expression and hormonal regulation of the KLK-L5 gene**

As shown in Figure 38, the KLK-L5 gene is primarily expressed in the salivary gland, stomach, uterus, trachea, prostate, thymus, lung, colon, brain, breast and thyroid gland. but, as is the case with other kallikreins, lower levels of expression are found in some other tissues (testis, pancreas, small intestine, spinal cord). In order to verify the RT-PCR specificity, the PCR products were cloned and sequenced. The three splice forms of the gene were expressed in most of these tissues. However, the relative abundance of each form was different among tissues (Figure 38).

In order to investigate whether the KLK-L5 gene is under steroid hormone regulation, two breast cancer cell lines (BT-474 and T-47D) and a prostate cancer cell line (LNCaP) were used as models. In LNCaP cells, the gene was up-regulated only by androgen and progesterin. Only in this cell line all 3 isoforms were expressed. In BT-474 cells, KLK-L5 was found to be up-regulated, at the mRNA level, by estrogen and androgen, and to a lesser extent by the progesterin. The rank of potency was estrogen>androgen>progesterin. However, the rank of potency for the T-47D cell line was androgen>progesterin>estrogen. In both of these cell lines, only the short isoform (related protein-2) was present (Figure 39).

#### **KLK-L5 is down regulated in breast cancer**

Expression of the KLK-L5 gene, at the mRNA level, was compared between seventeen breast cancer tissues and one normal breast tissue, by RT-PCR. The gene is not expressed at all in 12 tumors (Figure 40). In all breast tissues (normal and malignant) the short isoform (related protein-2) was predominant, with the exception of one tumor, which expressed only the classical form (Figure 40, lane 8). These results should be interpreted as preliminary, since the number of tumors and normal tissues tested is relatively small.

#### **Mapping and chromosomal localization of the KLK-L5 gene**

The knowledge of extensive genomic sequence on chromosome 19q13.3-q13.4 enabled the precise localization of 14 kallikrein-like genes and determination of the direction of transcription, as shown by the arrows in Figure 28. Only PSA and KLK2 transcribe from centromere to telomere; the rest of the genes are transcribed in the reverse direction. The KLK1 gene was found to be the most centromeric, and the KLK-L6 gene the most telomeric (KLK-L6; SEQ.ID. NO.65). KLK-L5 is 21.3 Kb centromeric to KLK-L4 (SEQ.ID.NO. 43) and 1.6 kb more telomeric to the trypsin-like serine protease gene (TLSP) (Figure 28).

#### **DISCUSSION**

As shown in Figure 34, kallikreins are characterized by the following common structural features: (a) All genes are formed of 5 coding exons and 4 intervening introns [some genes may have extra 5' untranslated exon(s)] (14, 35). (b) The exon lengths are usually comparable. (c) The intron phases are always conserved (I-II-I-0) (see legend of Figure 34 for definition of intron phases). (d) These genes are clustered in the same chromosomal region, without any intervening non-kallikrein-like genes (Figure 28). (e) The histidine residue of the catalytic triad of serine proteases is located near the end of the second coding exon; the aspartate residue in the middle of the third coding exon; and serine at the beginning of the

fifth coding exon. As shown in Figure 34, all these criteria apply to the newly identified KLK-L5 gene. Thus, KLK-L5 should be considered a new member of the kallikrein multigene family.

Serine proteases and kallikreins are synthesized as "preproenzymes" that contain an N-terminal signal peptide (pre-zymogen), followed by a short activation peptide and the enzymatic domain (41, 54). PreproPSA has 24 additional residues that constitute the pre-region (signal peptide, 17 residues), and the propeptide (7 residues) (67). The signal peptide directs the protein to and across the endoplasmic reticulum (ER). In the ER, the signal peptide is removed and the resulting proPSA is transported to the plasma membrane, where it is secreted. The hydrophobicity study (Figure 35) indicates that the amino terminal region of the KLK-L5 protein is harboring a signal peptide. Also, computer analysis of the amino acid sequence of KLK-L5 predicted a cleavage site between amino acids 17 and 18 (SQA-AT). Thus, the protein product is very likely a secreted protein.

The presence of alternatively spliced forms is not a unique feature of the KLK-L5 gene; several other kallikreins are known to be expressed in various alternatively spliced forms. In addition to the major 1.6-kb transcript, several RNA species are transcribed from the PSA gene (61). Furthermore, others (69, 70) have described several PSA isoforms. Retained introns and loss of complete exons have been reported in some of these forms. In addition, Riegman et al. reported the identification of two alternatively spliced forms of the human glandular kallikrein (KLK2) gene (62) and Liu et al. isolated three alternative forms of the same gene (68). A novel transcript of the tissue kallikrein gene was isolated from the colon (63). Neuropsin, a recently identified kallikrein-like gene, was found to have two alternatively spliced forms in addition to the major form (35, 64). Here, the cloning of the classical kallikrein form and two unique splice forms of the KLK-L5 gene are described. Because the classical form and the splice forms all have the same 5' sequence required for translation, secretion and activation as do other kallikreins, i.e. a 5' leader sequence, a signal peptide, and a proregion, it is reasonable to assume that all three mRNA forms should produce a secreted protein. Preliminary findings identifying forms of KLK-L5 predominant in certain tissues are presented in Figures 35 and 40.

The preliminary results indicate that KLK-L5 is up-regulated by steroid hormones in breast and prostate cancer cell lines (Figure 39). These results are not surprising, since many other kallikrein genes are also regulated by steroid hormones. The differences in the rank of potency of steroid hormones among different cell lines could be attributed to differences in the abundance of the steroid hormone receptors between them as described elsewhere.

In conclusion, a new member of the human kallikrein gene family, KLK-L5, has been characterized which maps to the human kallikrein locus (chromosome 19q13.3-q13.4). This gene has two related splice forms in addition to the main form. KLK-L5 is expressed in a variety of tissues, appears to be down-regulated in breast cancer and its expression is influenced by steroid hormones. Since a few other kallikreins are already used as valuable tumour markers, KLK-L5 may also find a similar clinical application.

#### Example 7

Using the Materials and Methods substantially as set out in Example 6, the present inventors

identified another novel gene of the kallikrein multigen family referred to as KLK-L6. The full structure of the KLK-L6 gene is shown in Figure 41. Exons 1, 2, 3, 4, 5, 6, and 7 are at nucleic acids 1172-1281; 2561-2695; 2781-2842, 3714-3885; 5715-5968; 6466-6602; and 7258-7520. The nucleic acid sequence of the KLK-L6 gene is also shown in SEQ.ID.NO. 65 and amino acid sequences for the KLK-L6 protein are shown in SEQ.ID. Nos. 66 and 67. (See also GenBank Accession # AF161221).

Figure 42 shows a comparative hydrophobicity analysis of KLK-L6 and the prostate-specific antigen (PSA). The amino terminal regions of both genes are quite hydrophobic indicating that this region of KLK-L6 is possibly harboring a signal peptide analogous to PSA

Multiple alignment of KLK-L6 was carried out using the Clustal X software program as described herein (Figure 43).

To predict the phylogenetic relatedness of the KLK-L6 protein with other serine proteases, the amino acid sequences of the kallikrein proteins were aligned together using the "Clustal X" multiple alignment program and a distance matrix tree was predicted using the Neighbor-joining/UPGMA and Protpars parsimony methods. Figure 44 shows the phylogenetic analysis which separated the classical kallikreins (hK1, hK2, and PSA) and placed KLK-L6 in a separate group.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. All modifications coming within the scope of the following claims are claimed.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

**FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION**

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TABLE 1. Predicted exons of the putative gene KLK-L1. The translated protein sequences of each exon (open reading frame) are shown.

Exon No.	Putative coding region <sup>a</sup>	No. of bases	Translated protein sequence	EST match <sup>3</sup>	Intron phase <sup>4</sup>	Stop codon <sup>5</sup>	Catalytic triad <sup>6</sup>	Exon prediction program <sup>7</sup>	
	From(bp)	To (bp)							
2	2263	2425	163	SLVSGSCQINGEDCSHPSPWQAALVMENELFCSGV LVHPQWVLSAAHCFQ	+	II	-	H	A,B,D
3	2847	3109	263	NSYTLGLHLSEADQPGSQMVEASLSYRHPPEYNRL LANDLMLIKLDESVSSEDITRSISIASQCTAGNSCLVSG WGLLANGELT	+	I	-	D	A,B,C,D
4	3180	3317	137	GRMFTVLQCVNVSVVSEEVCSKLYDPLYPHSMFCAGG GDDKQDSCN	+	0	-	-	A,B,C,D
5	4588	4737	150	GDSGGPLICNGYLQGLVSYFGKAPCGGVGPVYTNLC KFTIEWIKTVQAS	+	-	+	S	A,B,C

1. Conventional numbering of exons in comparison to the five coding exons of PSA, as described in Ref. 14.

2. Nucleotide numbers refer to the related contig.

3. (+) = &gt;95% homology with published human EST sequences.

4. Intron phase: 0—the intron occurs between codons; I—the intron occurs after the first nucleotide of the codon;

II—the intron occurs after the second nucleotide of the codon.

5. (+) denotes the exon containing the stop codon.

6. H=histidine, D=aspartic acid, S=s erine. The aminoacids of the catalytic triad are bold and underlined.

7. A = GeneBuilder (gene analysis), B = GeneBuilder (exon analysis), C = Grail 2,

D = GENEID-3

TABLE 2. Predicted exons of the putative gene KLK-L2. The translated protein sequences of each exon (open reading frame) are shown.

Exon No. <sup>1</sup>	Putative coding sequence <sup>2</sup> From(bp) To(bp)	No. of bases	Translated protein sequence	EST match <sup>3</sup>	Intron phase <sup>4</sup>	Step codon <sup>5</sup>	Catalytic triad <sup>6</sup>	Exon prediction program <sup>7</sup>
1	15,361 15,433	73	MATARPPVXWVLCALITALLLOVT	+	I	-	-	-
2	17,904 18,165	262	EHVLANNVSCDHPSTNTPVSGSNQDLGAGAGEDARDSDSSRIIN GSDCDMHTQPWQAALLRPNQLYCGAVLVHPQWLLTAAHCRK K	+	II	-	H	A,B,C,D
3	18,903 19,159	257	VFRVLGHVLSLPVYESGQQMFQGVKSIHPHPCYSHPOHNDMLLI KLNRRIPTKDYRPNVSSHCPAGTKCLYSGWGTTKSPQ	+	I	-	D	C,D
4	19,245 19,378	134	VHFRKVLQCLNISVLSQKRCEAYPRQIDTIFCAQDKAGRDSC Q	+	0	-	-	B,C
5	24,232 24,384	153	GDGGPVVYVNGSLQGLVSWGDPYCARPNRPQVYTNLCKFTKWI QETIQANS	+	-	+	S	A,B,C

\* All footnotes same as Table 1

TABLE 3. Predicted exons of the putative gene KLK-L3. The translated protein sequences of each exon (open reading frame) are shown

Exon No. <sup>1</sup>	Putative coding region <sup>2</sup>	No. of bases	Translated protein sequence	EST match <sup>3</sup>	Intron phase <sup>4</sup>	Stop codon <sup>5</sup>	Catalytic triad <sup>6</sup>	Exon prediction program <sup>7</sup>
1	From(bp) 70,473 To(bp) 70,584	112	VHFTPINHRGGPNEEGDMAYHKEALDAGCTQDP	-	I	-	-	A,B,C,D
2	70,764 70,962	199	ACSSLTPLSLPTPGHGWADTRAGAECCRPNSQPWQAGLF HLTRLFCGATLISDRWLLTAACHCK	+	II	-	H	A,B,C,D
3	73,395 73,687	293	PLTSEACPSRYLWVRLGEHLWKWEGPEQLFRVYDFRHP GFNKDLASNDHNDIMILRLPQARLSPAVQPLNLSQTCV SFGMQCLISGWGVSSPK	+	I	-	D	A,B,C,D
4	76,305 76,441	137	ALFFVTLQCANISILENKLCHWAYPGHISDSMLCAQLWEG GRGSCQ	+	0	-	-	A,B,C,D
5	76,884 77,633	749	GPQGPPLVQNGTLAGVYVSGAEPSCRFRPRPAPVYTSVCHYL DWIQEIMEN	-	-	+	S	A,B

\* All footnotes same as Table 1

TABLE 4. Predicted exons of the putative gene KLIK-L4. The translated protein sequences of each exon (open reading frame) are shown

Exon No. <sup>1</sup>	Putative coding region <sup>2</sup> From (bp) To (bp)	No. of bases	Translated protein sequence	EST match <sup>3</sup>	Intron phase <sup>4</sup>	Stop codon <sup>5</sup>	Catalytic triad <sup>6</sup>	Exon prediction program <sup>7</sup>
2	24,945 25,120	176	ESSKVLNTNGTSGFLFGYTCFPHSQPWAALLVQGRLL CGVLVHPKWVLTAAHCLKE	+	II	-	H	C
3	25,460 25,728	269	GLKYYLGKIALGRVEAGEOYREVVISHPHPEYRRSPFHL NHDHIMLELQSPVQLTGYIQLPLSHNNRLPTGTCRV SWGTTTSPQ	+	I	-	D	A,B,C,D
4	26,879 27,015	137	VNYPKTLQCANQLRSDEECRQYVFGKITDNNMLCAGTKE GGKDSCE	+	0	-	-	A,B,C,D
5	28,778 28,963	189	GDSSGPLYCNRTLYGVISWGDFFGCGQDPFGVYTRYRVRY VLWIRETRKYETQQQKWLKGPQ	+	-	+	S	A,B,C

\* All footnotes same as Table 1

TABLE 5. Predicted exons of the putative gene KLK-L5. The translated protein sequences of each exon (open reading frame) are shown.

Exon No. <sup>1</sup>	Punative coding region <sup>2</sup> From(bp) To(bp)	No. of bases	Translated protein sequence	EST match <sup>3</sup>	Intron phase <sup>4</sup>	Stop codon <sup>5</sup>	Catalytic triad <sup>6</sup>	Exon prediction program
2	1588 1747	160	LSQAATPKIFNGTECGRNSQFWQYGLFEGTSLRCGGV LIDHRWYL TAAHCSSG	-	II	-	H	A,B,C
3	3592 3851	260	SRVWVRLGEHLSQLDWTQIRHSGFSVTHFOYLGAS TSHEHDLRLRLRLPVRVTSSVQPLPFDNCATAGTEC HVSQWQHTNHPR	+	I	-	D	A,B,C,D
4	4806 4939	134	NPFDDLOCLNLSIVSHATCHGVVPGRTSNMVCAGG VFQDQACQ	+	0	-	-	A,B,C,D

\* All footnotes same as Table 1

TABLE 6. Homology between the predicted amino acid sequences of the newly identified putative genes and protein sequences deposited in Genbank

No.	Gene identity	Homologous known protein	Identity% (number of amino acids)
1	KLK-L1	• Human stratum corneum chymotryptic enzyme	44(101/227)
		• Rat kallikrein	40( 96/237)
		• Mouse glandular kallikrein K22	39( 94/236)
		• Human glandular kallikrein	38( 93/241)
		• Human prostatic specific antigen	37( 91/241)
		• Human protease M	37( 87/229)
2	KLK-L2	• Human neuropsin	48(106/219)
		• Human stratum corneum chymotryptic enzyme	47(103/216)
		• Human protease M	45( 99/219)
		• Human trypsinogen I	45(100/221)
		• Rat trypsinogen	44( 98/220)
3	KLK-L3	• Human neuropsin	44(109/244)
		• Rat trypsinogen 4	39( 95/241)
		• Human protease M	38( 98/253)
		• Human glandular kallikrein	37( 94/248)
		• Human prostatic specific antigen	36( 89/242)
4	KLK-L4	• Human protease M	52(118/225)
		• Human neuropsin	51(116/225)
		• Mouse neuropsin	51(116/226)
		• Human glandular kallikrein	48(113/234)
		• Human prostatic specific antigen	47(108/227)
5	KLK-L5	• Human neuropsin	44( 81/184)
		• Rat trypsinogen I	42( 76/178)
		• Rat trypsinogen II	42( 75/178)
		• Human protease M	41( 73/178)



**Table 7.** Expressed sequence tags with >95% homology to exons of the prostate/CLK-L1 gene.

GenBank #	Source	Tissue	homologous exons
AA551449	I.M.A.G.E.	prostate	3,4,5
AA533140	I.M.A.G.E.	prostate	4,5
AA503963	I.M.A.G.E.	prostate	5
AA569484	I.M.A.G.E.	prostate	5
AA336074	TIGR	endometrium	2,3

**Table 8.** Primers used for reverse transcription-polymerase chain reaction (RT-PCR) analysis of various genes.

Gene	Primer name	Sequence <sup>1</sup>	Product size (base pairs)
Protease (KLK-L1)	RS	TGACCCGCTGTACCACCCA	278
	RAS	GAATTCCTTCCGCAGGATGT	
pS2	PS2S	GGTGATCTGCGCCCTGGTCCT	328
	PS2AS	AGGTGTCCGGTGGAGGTGGCA	
PSA	PSAS	TGCGCAAGTTCACCCTCA	754
	PSAAS	CCCTCTCCTTACTTCATCC	
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT	372
	ACTINAS	TCTCCTTAATGTCACGCACGA	

1. All nucleotide sequences are given in the 5'→3' orientation.

**Table 9.** Tissue expression of prostate/CLK-L1 by RT-PCR analysis

Expression level			
High	medium	low	No Expression
Prostate	Mammary gland	Salivary glands	Stomach
Testis	Colon	Lung	Heart
Adrenals	Spinal cord	Brain	Spleen
Uterus		Bone marrow	Placenta
Thyroid		Thymus	Liver
		Trachea	Pancreas
		Cerebellum	Kidney
			Fetal brain
			Fetal liver
			Skeletal muscle
			Small intestine

Table 10. EST clones with &gt;95% homology to exons of KLK-L2

GENBANK #	Tissue of Origin	I.M.A.G.E. ID	Homologous exons
W73140	Fetal heart	344588	4,5
W73168	Fetal heart	344588	3,4,5
AA862032	Squamous cell carcinoma	1485736	4,5
AI002163	Testis	1619481	3,4,5
N80762	Fetal lung	300611	5
W68361	Fetal heart	342591	5
W68496	Fetal heart	342591	5
AA292366	Ovarian tumor	725905	1,2
AA394040	Ovarian tumor	726001	5

Table 11. Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Gene	Primer name	Sequence <sup>1</sup>	Product size (base pairs)
KLK-L2	KS	GGATGCTTACCCGAGACAGA	342
	KAS	GCTGGAGAGATGAACATTCT	
pS2	PS2S	GGTGATCTGCGCCCTGGTCCT	328
	PS2AS	AGGTGTCCGGTGGAGGTGGCA	
PSA	PSAS	TGCGCAAGTTCACCCCTCA	754
	PSAAS	CCCTCTCCTTACTTCATCC	
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT	372
	ACTINAS	TCTCCTTAATGTCACGCACGA	
KLK-L2	R1	CCGAGACGGACTCTGAAAACCTTCTTCC	
	R2	TGAAAACCTTCTTCTCCTGCAGTGGGCGGC	

1. All nucleotide sequence are given in the 5' 3' orientation.

Table 12. Tissue expression of KLK-L2 by RT-PCR analysis.

sion level			
high	Medium	low	No Expression
Brain	Salivary gland	Uterus	Stomach
Mammary gland	Fetal brain	Lung	Adrenal gland
Testis	Thymus	Heart	Colon
	Prostate	Fetal liver	Skeletal muscle
	Thyroid	Spleen	
	Trachea	Placenta	
	Cerebellum	Liver	
	Spinal cord	Pancreas	
		Small intestine	
		Kidney	
		Bone marrow	

TABLE 13 . Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Gene	Primer name	Sequence <sup>1</sup>
KLK-L3	L3-F1	CATGCAGTGTCTCATCTCAG
	L3-F2	CATGGAGGAGGAAGGAGATG
	L3-R1	CTTCGGCCTCTCTTGGTCTT
PSA	PSAS	TGCGCAAGTTCACCCTCA
	PSAAS	CCCTCTCCTTACTTCATCC
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT
	ACTINAS	TCTCCTTAATGTCACGCACGA

1. All nucleotide sequence are given in the 5' → 3' orientation.

TABLE 14. Primers used for gene-specific PCR amplification of the kallikrein genes using DNA as a template.

Primer name	Sequence <sup>1</sup>	Coordinates	GenBank accession #	Gene name
Z1S	GACCCTGACATGTGACATCTA	979-999	U62801	Zyme
Z1AS	GCCACTGCCTGATGGAGACTG	1422-1402		
GL3-F1	AACATCAGCATCCTGGAGAA	7324-7343	AF135026	KLK-L3
LL3-R1	CTTCGGCTCTCTTGGTCTT	8051-8060		
L2-1	GGGTCAGAGCTGCAGAGAAG	11104-11123	AF135028	KLK-L2
L2-2	GGGCCTGTCGTCTGCAATGG	11522-11541		
KLK-L1	ATGGCCACAGCAGGAAATCC	1411-1430	AF113141	KLK-L1
	GGTCACTTGTCTGCGCAGAC	1990-2019		
PS	CCCAACCCTGTGTTTTTCTC	3634-3653	M33105	PSA <sup>2</sup>
PAS	GGCCCTCCTCCCTCAGA	4143-4118		
K1S	ATCCCTCCATTCCCCTCTTT	2-22	M18157	KLK1 <sup>3</sup>
K1AS	CACATACAATTCTCTGGTTC	324-30		
K2S	AGTGACACTGTCTCAGAATT	131-150	AF024605	KLK2 <sup>4</sup>
K2AS	CCCCAATCTCACCAGTGCAC	580-561		
NS	GCTTCCTTACCGCTGTGCT	552-570	AF055481	NES1 <sup>5</sup>
NAS	CACCTCTGGCAAGGGTCCTG	763-744		

1. all nucleotide sequences are given in the 5'→3' orientation
2. prostate specific antigen
3. human renal kallikrein
4. human glandular kallikrein
5. normal epithelia cell-specific 1 gene.



TABLE 15. Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Gene	Primer name	Sequence <sup>1</sup>
KLK-L4	L4-F1	AACTCTACAATGTGCCAACA
	L4-R1	TTATTGTGGCCCTTCAACC
	L4-R3	GGATGGTCCATTTATAGGAC
	L4-A	AGGCTGCCCTACTAGTGCAA
	L4-B	ATATTGCCTAGGTGGATGTG
	L4-D	AAGACTTCAAGGAGCCAAGC
	L4-E	GACCTTCACCTCCCAAAAT
	L4-X1	CTAGTGATCGCCTCCCTGAC
pS2	PS2S	GGTGATCTGCGCCCTGGTCCT
	PS2AS	AGGTGTCCGGTGGAGGTGGCA
PSA	PSAS	TGCGCAAGTTCACCCTCA
	PSAAS	CCCTCTCCTTACTTCATCC
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT
	ACTINAS	TCTCCTTAATGTCACGCACGA

1. All nucleotide sequence are given in the 5'→3' orientation.

TABLE 16. EST clones with &gt;95% homology to exons of KLK-L4

GenBank #	Tissue of origin	I.M.A.GE. ID
AA399955 AA401397	Testis	743113
AA846771	Testis	1392889
AI002101	Testis	1619045
AI032327	Testis	1644236

TABLE 17. Primers used for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Gene	Primer name	Sequence <sup>1</sup>
KLK-L5	KLK-L5-F1	TCAGCCAGGCAGCCACACCG
	KLK-L5-R1	TTGGTGATGCCCCAGCCTGA
	L5-F2	CCACACCGAAGATTTTCAAT
	L5-R2	GCCCCTCCTTCATTATA
PSA	PSAS	TGCGCAAGTTCACCTCA
	PSAAS	CCCTCTCCTTACTTCATCC
Actin	ACTINS	ACAATGAGCTGCGTGTGGCT
	ACTINAS	TCTCCTTAATGTCACGCACGA

1. All nucleotide sequence are given in the 5'→3' orientation.

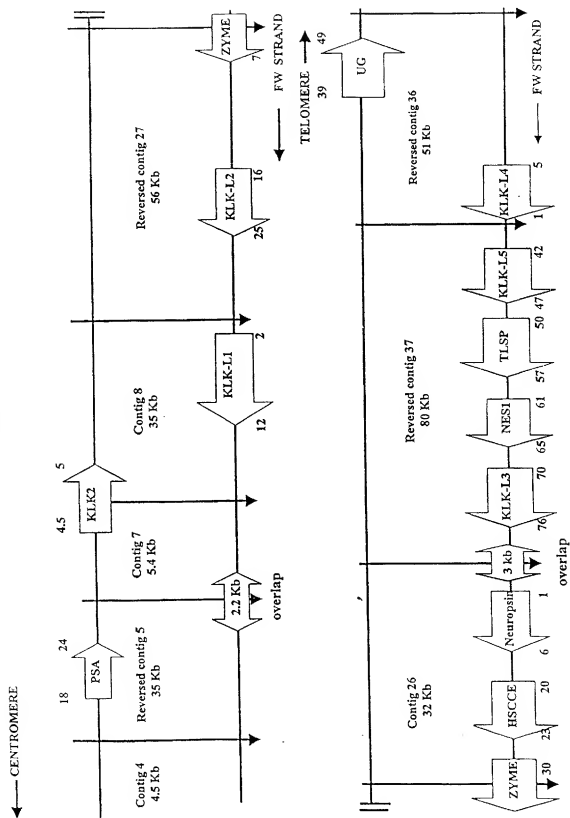
We Claim:

1. An isolated KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 nucleic acid molecule of at least 30 nucleotides which hybridizes to SEQ ID NO. 1, 13, 21, 43, 56, or 65, respectively, or the complement of SEQ ID NO. 1, 13, 21, 43, 56, or 65, under stringent hybridization conditions
2. An isolated nucleic acid molecule which comprises:
  - (i) a nucleic acid sequence encoding a protein having substantial sequence identity with an amino acid sequence of a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively;
  - (ii) a nucleic acid sequence encoding a protein comprising an amino acid sequence of a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively;
  - (iii) nucleic acid sequences complementary to (i);
  - (iv) a degenerate form of a nucleic acid sequence of (i);
  - (v) a nucleic acid sequence capable of hybridizing under stringent conditions to a nucleic acid sequence in (i), (ii) or (iii);
  - (vi) a nucleic acid sequence encoding a truncation, an analog, an allelic or species variation of a protein comprising an amino acid sequence of a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein as shown in SEQ.ID.NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67, respectively; or
  - (vii) a fragment, or allelic or species variation of (i), (ii) or (iii).
3. A purified and isolated nucleic acid molecule of the invention comprises:
  - (i) a nucleic acid sequence comprising the sequence of SEQ.ID.NO. 1, 13, 21, 43, 56, or 65 wherein T can also be U;
  - (ii) nucleic acid sequences complementary to (i), preferably complementary to the full nucleic acid sequence of SEQ.ID.NO. 1, 13, 21, 43, 56, or 65;
  - (iii) a nucleic acid capable of hybridizing under stringent conditions to a nucleic acid of (i) or (ii) and preferably having at least 18 nucleotides; or
  - (iv) a nucleic acid molecule differing from any of the nucleic acids of (i) to (iii) in codon sequences due to the degeneracy of the genetic code.
4. An isolated nucleic acid molecule which encodes a protein which binds an antibody of a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein.
5. A regulatory sequence of an isolated nucleic acid molecule as claimed in any of the preceding claims fused to a nucleic acid which encodes a heterologous protein.
6. A vector comprising a nucleic acid molecule of any of the preceding claims.
7. A host cell comprising a nucleic acid molecule of any of the preceding claims.

8. An isolated KLK-L1 protein comprising an amino acid sequence of SEQ. ID. NO. 2 or 3.
9. An isolated KLK-L2 protein comprising an amino acid sequence of SEQ. ID. NO. 14.
10. An isolated KLK-L3 protein comprising an amino acid sequence of SEQ. ID. NO. 22 or 23.
11. An isolated KLK-L4 protein comprising an amino acid sequence of SEQ. ID. NO. 44 or 45.
- 5 12. An isolated KLK-L5 protein comprising an amino acid sequence of SEQ. ID. NO. 57, 58, 59, or 60.
13. An isolated KLK-L6 protein comprising an amino acid sequence of SEQ. ID. NO. 66 or 67.
14. An isolated protein having at least 65% amino acid sequence identity to an amino acid sequence of SEQ. ID. NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67.
15. A method for preparing a protein as claimed in any of the preceding claims comprising:
  - 10 (a) transferring a vector as claimed in claim 6 into a host cell;
  - (b) selecting transformed host cells from untransformed host cells;
  - (c) culturing a selected transformed host cell under conditions which allow expression of the protein; and
  - (e) isolating the protein.
- 15 16. A protein prepared in accordance with the method of claim 15.
17. An antibody having specificity against an epitope of a polypeptide as claimed in claim 8, 9, 10, 11, 12, or 13.
18. An antibody as claimed in claim 17 labeled with a detectable substance and used to detect the protein in biological samples, tissues, and cells.
- 20 19. A probe comprising a sequence encoding a protein as claimed in claim 8, 9, 10, 11, 12, or 13, or a part thereof.
20. A method of diagnosing and monitoring conditions mediated by a protein as claimed in claim 8, 9, 10, 11, 12, or 13, by determining the presence of a nucleic acid molecule encoding the protein as claimed in any of the preceding claims or determining the presence of the protein.
- 25 21. A method as claimed in claim 20 wherein the condition is cancer.
22. A method for identifying a substance which associates with a protein as claimed in claim 8, 9, 10, 11, 12, or 13 comprising (a) reacting the protein with at least one substance which potentially can associate with the protein, under conditions which permit the association between the substance and protein, and (b) removing or detecting protein associated with the substance, wherein detection of associated protein and substance indicates the substance associates with the protein.
- 30 23. A method for evaluating a compound for its ability to modulate the biological activity of a protein as claimed in claim 8, 9, 10, 11, 12, or 13 comprising providing a known concentration of the protein with a substance which associates with the protein and a test compound under conditions which permit the formation of complexes between the substance and protein, and removing and/or detecting complexes.
- 35 24. A method for detecting a nucleic acid molecule encoding a protein comprising an amino acid sequence of SEQ. ID. NO. 2, 3, 14, 22, 23, 44, 45, 57, 58, 59, 60, 66, or 67 in a biological sample comprising the steps of:

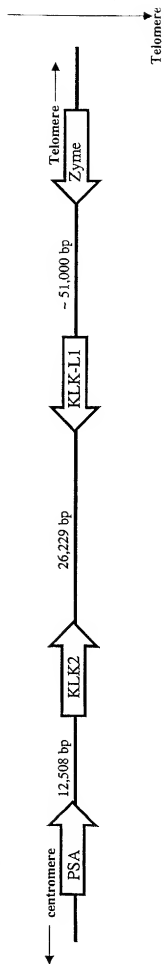
- (a) hybridizing a nucleic acid molecule of claim 2 to nucleic acids of the biological sample, thereby forming a hybridization complex; and
- (b) detecting the hybridization complex wherein the presence of the hybridization complex correlates with the presence of a nucleic acid molecule encoding the protein in the biological sample.
- 5 25. A method as claimed in claim 24 wherein nucleic acids of the biological sample are amplified by the polymerase chain reaction prior to the hybridizing step.
26. A method for treating a condition mediated by a protein as claimed in claim 8, 9, 10, 11, 12, or 13 comprising administering an effective amount of an antibody as claimed in claim 17 or a substance or compound identified in accordance with a method claimed in claim 22 or 23.
- 10 27. A method as claimed in claim 26 wherein the condition is cancer.
28. A composition comprising one or more of a nucleic acid molecule or protein claimed in any of the preceding claims, or a substance or compound identified using a method as claimed in any of the preceding claims, and a pharmaceutically acceptable carrier, excipient or diluent.
- 15 29. Use of one or more of a nucleic acid molecule or protein claimed in any of the preceding claims, or a substance or compound identified using a method as claimed in any of the preceding claims in the preparation of a pharmaceutical composition for treating a condition mediated by a protein as claimed in any of the preceding claims.
30. A transgenic non-human mammal which does not express a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein as claimed in claim 8, 9, 10, 11, 12, or 13, respectively, resulting in a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein associated pathology, respectively.
- 20 31. A transgenic animal assay system which provides a model system for testing for an agent that reduces or inhibits an a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein associated pathology comprising
- 25 (a) administering the agent to a transgenic non-human animal as claimed in claim 26; and
- (b) determining whether said agent reduces or inhibits a KLK-L1, KLK-L2, KLK-L3, KLK-L4, KLK-L5, or KLK-L6 protein associated pathology in the transgenic non-human animal relative to a transgenic non-human animal of step (a) which has not been administered the agent.
- 30

FIGURE 1



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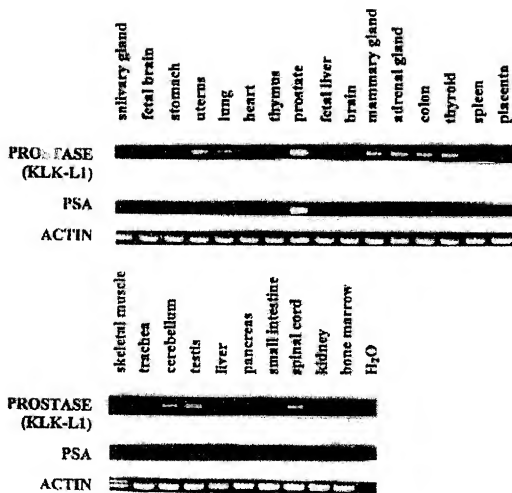
FIGURE 2





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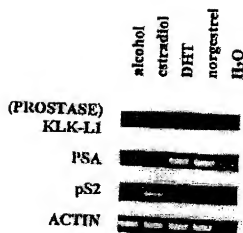
FIGURE 3



**FIGURE 4**

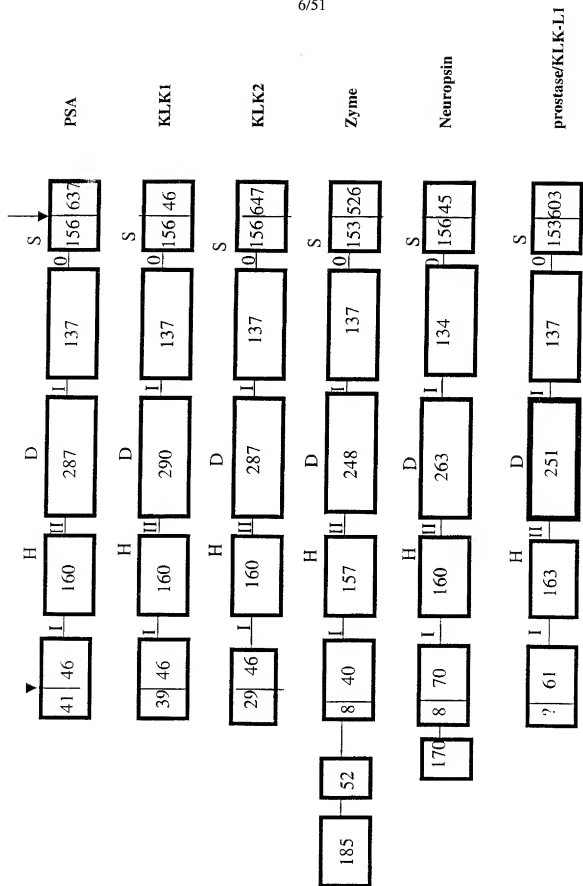
TGACCCGCTG TACCACCCCA GCATGTTCTG CGCCGGCGGA GGGCAAGACC  
AGAAGGACTC CTGCAACGGT GACTCTGGGG GGCCCTGAT CTGCAACGGG  
TACTTGCAGG GCCTTGTGTC TTTCGGAAAA GCCCGTGTG GCCAAGTTGG  
CGTGCCAGGT GCCTACACCA ACCTCTGCAA ATTCACTGAG TGGATAGAGA  
AAACCGTCCA GGCCAGTTAA CTCTGGGGAC TGGGAACCCA TGAAATTGAC  
CCCCAAATAC ATCCTGCGGA AGGAATTC

FIGURE 5



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FIGURE 6

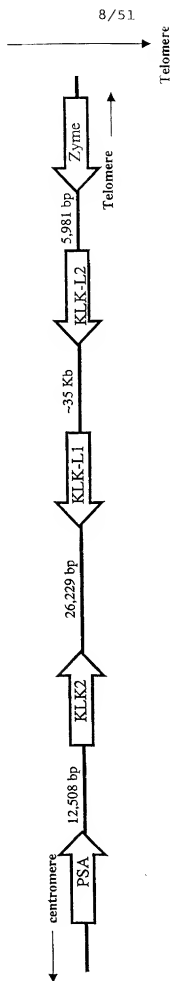


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## FIGURE 7

(ATG)GCTACAGCAAGACCCCCCTGGATGTGGGTGCTCTGTGCTCTGATCACAGCCT  
 M A T A R P P W M W V L C A L I T A  
 TGCTTCTGGGGTTCACAGGgagaccaga-----intron 1-----tcccgg  
 L L L G V T  
 AGCATGTTCTCGCCAACAATGATGTTTCTGTGACCACCCCTCTAACACCGTGCCC  
 E H V L A N N D V S C D H P S N T V P  
 TCTGGGAGCAACCAGGACCTGGGAGCTGGGGCCGGGAAGACGCCCGTCCGAT  
 S G S N Q D L G A G A G E D A R S D  
 GACAGCAGCAGCCGCATCAATGGATCCGACTGCGATATGCACACCCAGCCGT  
 D S S S R I I N G S D C D M H T Q P  
 GGCAGGCCGCGCTGTTGCTAAGGCCCAACAGCTCTACTGCGGGGGCGGTGTTGGT  
 W Q A A L L L R P N Q L Y C G A V L V  
 GCATCCACAGTGGCTGCTACGGCCGCCCACTGCAGGAAGAAgagagtgga-----  
 H P Q W L L T A A H C R K K  
 -----intron 2-----tcttctcagAGTTTTCAGAGTCCGTCCT  
 V F R V R L  
 CGGCCACTACTCCCTGTCAACAGTTTATGAATCTGGGCAGCAGATGTTCCAGGGG  
 G H Y S L S P V Y E S G Q Q M F Q G  
 GTCAAATCCATCCCCACCTGGCTACTCCCAACCTGGCCACTTAACGACCTCAT  
 V K S I P H P G Y S H P G H S N D L M  
 GCTCATCAAATGAACAGAAAGATTCTGCCACTAAAGATGTCAGACCCATCAAC  
 L I K L N R R I R P T K D V R P I N  
 GTCTCTCTCATTGTCCCTCTGCTGGGACAAAGTGCTTGGTGTCTGGCTGGGGGAC  
 V S S H C P S A G T K C L V S G W G T  
 AACCAAGAGCCCCAAGGtgagtgccaggl-----intron 3-----tgacag  
 T K S P Q  
 TGCACTTCCCTAAGGTCTCCAGTGCTTGAATATCAGCGTGCTAAGTCAGAAAAAG  
 V H F P K V L Q C L N I S V L S Q K R  
 GTGCGAGGATGCTTACCCGAGACAGATAGATGACACCATGTTCTGCGCCGGTGAC  
 C E D A Y P R Q I D D T M F C A G D  
 AAAGCAGGTAGAGACTCTGCCAGgagagacacc-----intron 4-----  
 ag  
 K A G R D S C Q  
 GGTGATTCTGGGGGGCCTGTGCTGCAATGGCTCCCTGCAGGGACTCGTGTCTCT  
 G D G G P V V C N G S L Q G L V S  
 GGGGAGATTACCTTGTGCCCGCCCAACAGACGGGTGTCTACACGAACCTCTG  
 W G D Y P C A R P N R P G V Y T N L C  
 CAAGTTCAACAGTGATGCCAGGAAACCATCCAGGCCAACTCCTGAGTCAATCC  
 CA  
 K F T K W I Q E T I Q A N S  
 GGACTCAGCACACCGGCATCCCCACCTGTGACGGGACAGCCCTGACACTCCTTT  
 CAGACCCCTCATTCCTTCCAGAGATGTTGAGAATGTTCTCTCTCCAGCCCTGAC  
 CCGATGTCTCTGGACTCAGGGTCTGCTTCCCCACATGGGGCTGACCGTGTCTCT  
 CTAGTTGAACCTTGGGAACAATTTCCAAAACTGTCCAGGGCGGGGGTGTGCTCTC  
 AATCTCCTCGGGGCACTTTTCATCCTCAAGCTCAGGGCCATCCCTCTCTGTCAGCT  
 CTGACCCAAATTTAGTCCAGAAATAAACTGAGAAG

FIGURE 8



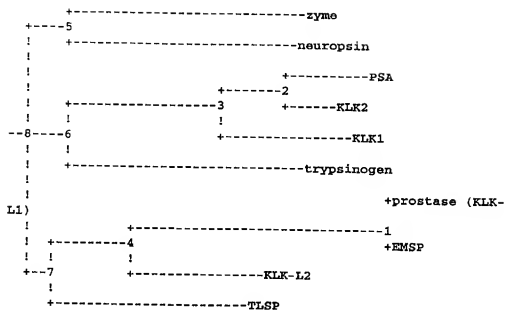
## FIGURE 9

protease	MATAGNFWGWFGLG----	YLILGVAGSLVSG-----	26	
EMSP	MATAGNFWGWFGLG----	YLILGVAGSLVSG-----	26	
KLK-L2	MATARPWMWVLCALITALLGVTEHVLANNVDSCDI	PSNTVPSSNQDLGAGAGEDARS	60	
zyme	-----	MKML-----	VVLSLTAANA-----	16
neuropsin	-MGRPRPRAAKTW-----	MFLLLGGAGAGH-----	S	26
TLSP	-----	MRLQ-----	LILLALATGLVG-----	17
PSA	-----	MWVVFV-----	LTLSTWIGAAPL-----	20
KLK2	-----	MWDLVLS-----	LALSVGCTGAUPL-----	20
KLK1	-----	MWFLVLC-----	LALSLGGTGAAPP-----	20
trypsinogen	-----	MNPLLI-----	LTFVAALAAFPD-----	19
		↑		
protease	--SCSQIINGEDCSPHSQPQAAALVM--	ENELFCSGVLVHPQVLSAAHC	FQNSYITIGLGI	83
EMSP	--SCSQIINGEDCSPHSQPQAAALVM--	ENELFCSGVLVHPQVLSAAHC	FQNSYITIGLGI	83
KLK-L2	DDSSSRIINGSDCDMTQFPQAAALLLRPNQLYCGAVLVHPQWLLTAAHCRKKVFRVLGH	120		
zyme	--EEQNKLVHGGPCDKTSHFYQAAALYT--	SGHLLCGGVLHPLVLTAAHCKKPNLOVPLGK	74	
neuropsin	RAQEDKVLGGHECQPHSQPQAAALFY--	GQQLCGGVLVGGMVLTAHCKKPKYTVRLGD	85	
TLSP	--GETRIIGFECQPHSQPQAAALFE--	KTRLLCGATLIAPRMLTAAHCLKPRYIVHLGQ	74	
PSA	--LTLRSIVGGWCEKHSQFPQVVLVAS--	RGRAVCGGVLVHPQWLLTAAHCIRNKSVILLGR	77	
KLK2	--IQSRIVGGWCEKHSQFPQVAVVYS--	HGWAHCGGVLVHPQWLLTAAHCLKKNQSVLGR	77	
KLK1	--IQSRIVGGWCEKHSQFPQAAALYH--	FSTFCGGILVHRQWLLTAAHCISDNQYLWLGR	77	
trypsinogen	--DDDKLVGGYNCEENSVFYQVLSLNS--	GYHFCGGSLINEQVLSGHCYSKRIQVRLGE	75	
	↑	↑	↑	↑
protease	HSLEADQEPGSMVEASLSVRHPEYN----	RP-----	LLANDLMLKLKDESVS--ESDT	131
EMSP	HSLEADQEPGSMVEASLSVRHPEYN----	RP-----	LLANDLMLKLKDESVS--ESDT	131
KLK-L2	YLSLPVYEGSQMFGQVKSIPHPGYS-----	HP-----	GHSNDLMLKLKLNRRIR--PTMD	168
zyme	HNLRO--RESSQEQSSVVRVAVHPDY----	DAA-----	SHDQDMLKLRLARPAK--LSEL	121
neuropsin	HSLNQ--KDFGBOEIPVQSIHPHCYN--	SSDVE-----	DHNHDMLKLQLRDQAS--LGSK	135
TLSP	HNLQK--EEGCEQTRTATESFPHPGFNNSLPNK-----	DHRND	IMLVKMASFVS--LTWA	125
PSA	HSLFH--PEDITGVFQVSHSFPHLYDMSSLKNNFLRRGDDSSH	DMLMLRLSEPAE--LTDA	135	
KLK2	HNLFE--PEDITGVFQVSHSFPHLYDMSSLKNNFLRRGDDSSH	DMLMLRLSEPAE--ITDV	135	
KLK1	HNLFD--DENTAQFVHVSSEFPHPGFNNSLLENHTRQEDYSH	DMLMLRLTEPADTITDA	136	
trypsinogen	HNIETV--LEGNEQFINAAKIRHPQYDKRTLNN-----	DIMLTKLSSRAV--INAR	122	
		↑	↑	↑
protease	IRISIASQCPTAGNSCLVSGWGLLANG--	RMPTVLQCVNVSVVSEEVCSKLYDPLVHPS	189	
EMSP	IRISIASQCPTAGNSCLVSGWGLLANG--	RMPTVLQCVNVSVVSEEVCSKLYDPLVHPS	189	
KLK-L2	VRPNVINSCHCSAGTKCLVSGWGTTKSPQVHFPPKVLQCLNLSVLQKRCEDAYPRQIDDT	228		
zyme	IQPLFLERCDSANTTSCILGWGKTADG--	DFPDITQCIYHLVSRSECHAYFGQITQ	179	
neuropsin	VKPSIADHCTQPGQKCTVSGWGTVTSPRENFPDILACAEVKIFPQKCEBAYPGNITDT	195		
TLSP	VRPNTLSGRCTVAGTSCILSGWGSTSPQLRLPHTLRACANTTIEHQKCEBAYPGNITDT	185		
PSA	VKVMDLPTQEPALGTTCYASGWSGIEPEEFLRTPKLCQVLDLHVISNDVCAQVHPQKVTIF	195		
KLK2	VKVLGLPTQEPALGTTCYASGWSGIEPEEFLRTPKLCQVLDLHVISNDVCAQVHPQKVTIF	195		
KLK1	VKVVELPTBEPVSGTCLASGWSGIEPEFSPDDLQCVDLKILPNDBCKAHVQKVTIF	196		
trypsinogen	VSTISLPTAPATGTGKCLISGWSGNTASSGADYPDELQCLDAPVLQSAKCEASYPGKITSN	182		
	↑	↑	↑	↑
protease	MFCAGGGHDQKDCSNGDSGGH	LICNGYLQGLVSPGKAPCGQVGVPGVYTNLCKFTWIEK	249	
EMSP	MFCAGGGHDQKDCSNGDSGGH	LICNGYLQGLVSPGKAPCGQVGVPGVYTNLCKFTWIEK	249	
KLK-L2	MFCAG--DKAGDRSCQ	GDSGGH	VVNCNGLSLGLVSWGDPYPCARNRNPVYTNLCCKFTKWIQE	287
zyme	MLCAGDEKYGKDCSQ	GDSGGH	LVCGDHLRGLVSWGNIPCGSKKEPGVYTNVCRYNLWJQK	239
neuropsin	MVCAGSSK--GADTCQ	GDSGGH	LVCGDGLGQITTSWGSDDPCGRSDKPGVYTNICRYLDMIKK	254
TLSP	MVCASVQEGGKDCSQ	GDSGGH	LVNCNQLGQITISWGDPCALITRKPQVYTKVKCYVDWIKD	255
PSA	MLCAGRWTKGKTS	GDSGGH	LVNCNGLVQITTSWGSSEPCALPERPSLYTKVHYRKMWD	255
KLK2	MLCAGLWTKGKDTCC	GDSGGH	LVNCNGLVQITTSWGSSEPCALPERPAVYTKVHYRKMWD	255
KLK1	MLCVGHLEGGKDTCC	GDSGGH	LMCDGVLQGVTSWGVYPCGTNRPSPVAVRVLVYKWKID	256
trypsinogen	MFCVGLFEGGKDCSQ	GDSGGH	VVNCNGLQGLVSWG--DGCAGKQNPVYTKVYNYVKWIKN	241
	↑	↑	↑	↑

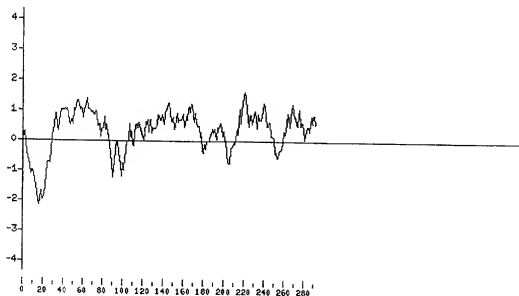
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## FIGURE 10

(A)



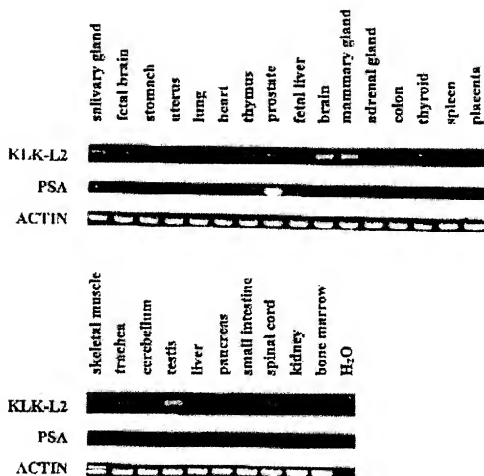
(B)





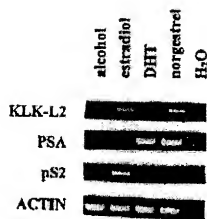
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FIGURE 11



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## FIGURE 12



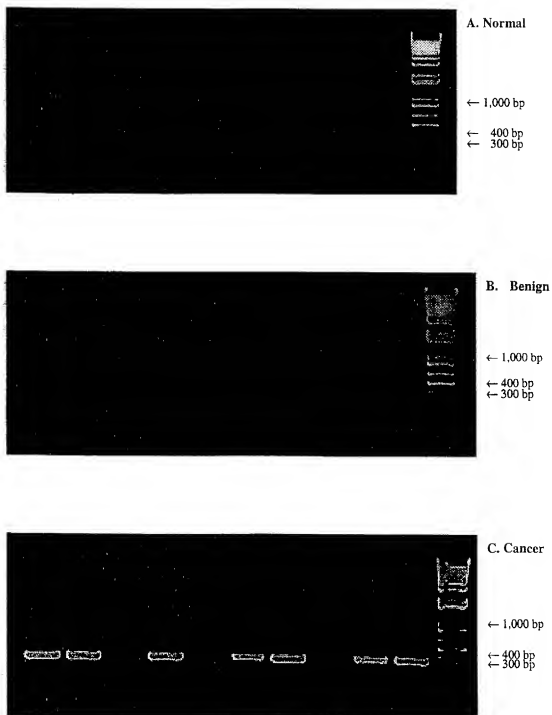
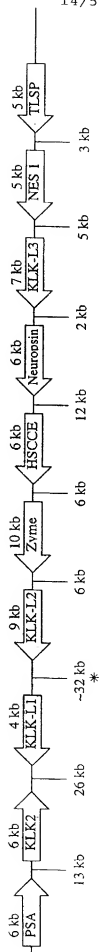
**FIGURE 13**

FIGURE 14



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## FIGURE 15

CAC TGG AC GGG GTG CAC GTT CAG GAT CCAG GTG CCC AGG GGT ATG AAG CTG GGA  
CTC

M K L G L

CTC TGT GCT CTG CTC TCT CTG CTG GCA G g t g a ... intron 1 . C f a g GG CAT  
GGC  
L C A L L S L L A G H  
G

TGG GCA GAC ACC CGT GCC ATC GGG GCC GAG GAA TGT CGC CCC AAC TCC  
CAG  
W A D T R A I G A E E C R C N S  
Q

CCT TGG CAG GCC GGC CTC TTC CAC CTT ACT CGG CTC TTC TGT GGG GCG  
ACC  
P W Q A G L F H L T R L F C G A T

CTC ATC AGT GAC CGC TGG CTG CTC ACA GCT GCC CAC TGC CGC A AG  
CC g t g a .....  
L I S D R W L L T A A H C R K P

intron 2 ..... g c a g G TAT CTG TGG GTC CGC CTT GGA GAG CAC CAC CTC TGG AAA  
Y L W V R L G E H H L W K

TGG GAG GGT CCG GAG CAG CTG TTC CGG GTT ACG GAC TTC TTC CCC CAC  
CCT  
W E G P E Q L F R V T D F F P H P

GGC TTC AAC AAG GAC CTC AGC GCC AAT GAC CAC AAT GAT GAC ATC ATG  
CTG  
G F N K D L S A N D H N D D I M L

ATC CGC CTG CCC AGG CAG GCA CGT CTG AGT CCT GCT GTG CAG CCC CTC  
AAC  
I R L P R Q A R L S P A V Q P L N

CTC AGC CAG ACC TGT GTC TCC CCA GGC ATG CAG TGT CTC ATC TCA GGC  
TGG  
L S Q T C V S P G M Q C L I S G W

GGG GCC GTG TCC AGC CCC AAG G g t a t ..... intron a c g CG CTG TTT CCA  
GTC  
G A V S S P K A L F P  
V

ACA CTG CAG TGT GCC AAC ATC AGC ATC CTG GAG AAC AAA CTC TGT CAC  
TGG  
T L Q C A N I S I L E N K L C H W

GCA TAC CCT GGA CAC ATC TCG GAC AGC ATG CTC TGT GCG GGC CTG TGG  
GAG  
A Y P G H I S D S M L C A G L W E

GGG GGC CGA GGT TCC TGC CAG G t g a ..... intron .. a c g GGT GAC TCT GGG  
GGC  
G G R G S C Q G D S G  
G

CCC CTG GTT TGC AAT GGA ACC TTG GCA GGC GTG GTG TCT GGG GGT GCT  
GAG  
P L V C N G T L A G V V S G G A E

## FIGURE 15 (CONT'D)

CCC TGC TCC AGA CCC CGG CGC CCC GCA GTC TAC ACC AGC GTA TGC CAC  
TAC

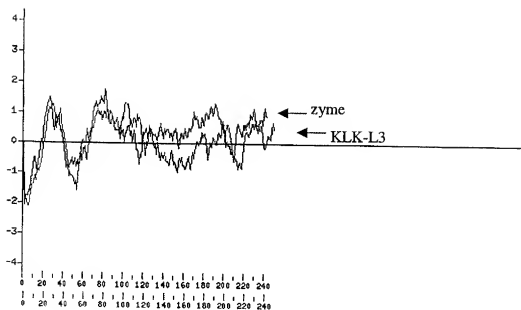
P C S R P R R P A V Y T S V C H Y

CTT GAC TGG ATC CAA GAA ATC ATG GAG AAC (TGA)  
L D W I Q E I M E N

GCCCGCGCGCCACGGGGGCACCTTGGAAGACCAAGAGAGGCCGAAGGGCACGGGGTA  
GGGGGTTCTCGTAGGGTCCCAGCCTCAATGGTTCCCGCCCTGGACCTCCAGCTGCCCTG  
ACTCCCTCTGGACACTAAGACTCCGCCCCCTGAGGCTCCGCCCCCTCACGGGTCAAGCA  
AGACACAGTCGCGCCCCCTCGGAACGGAGCAGGGACACGCCCTTCAGAGCCGTCTCTAT  
GACGTACCCGACAGCCATCACCTCCTTCTTGGAACAGCACAGCCTGTGGCTCCGCCCCA  
AGGAACCACTTACACAAAATAGCTCCGCCCTCGGAACTTTGCCCAAGTGGGACTTCCCC  
TCGGGACTCCACCCCTTGTGGCCCCGCTCCTTACCAGAGATCTCGCCCTCGTGATGT  
CAGGGGCGCAGTAGCTCCGCCACGTGGAGCTCGGGCGGTGTAGAGCTCAGCCCTTGTG  
GCCCGTCTCGGGCGTGTGCTGGGTTGAATCCTGGCGGAGACCTGGGGGGAAATTGAG  
GGAGGGTCTGGATACCTTTAGAGCCAATGCAACGGATGATTTTTCAGTAAACGGGGAAAC  
CTCA

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FIGURE 16



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## FIGURE 17

```

PSA -----MWVPVVFLTLSTVWIGAAPLI-LSRIVGGWCEKHSQPWQVLSASGRVAC
KLK2 -----MMDLVLSIALSVGCTGAVPLI-QSRIVGGWCEKHSQPWQVAVYSHGWAHC
KLK1 -----MWFLVLCIALSLGGTGAAPPI-QSRIVGGWCEKHSQPWQVAAALYHSTFQC
trypsinogen -----MNPLLILTFVAAALAAPFDD-DDKIVGGVNCENSVYPQVSLNS-GYNEF
KLK-L3 -----MKLGLLCALLSLLAGHWA--DTRATGAEECRPNSPQWQAALFHLTRLFC
TLSP -----MRI-LQILLLALATGLVGG--ETRIKIGFECKPHSPQWQAALFEKTRLIC
neuropsin -----MGRPRPRAAKTWMFLLLGGAAWAGHSRAQ-EDKVLGGHECQPHSPQWQAALFGQOQLLC
zyme -----MKK--LMVVLSLIAAAWAE-ONKLVHGGPCDKTSHPHYQAALYTSGHLLC
HSCCE ---MARSLLLPLQILLLSLALETAGEEAGG--DKIIGDAPCARGSHPWQVALLSCNQLHC
protease ---MA-TAGNPMGWFLGYLILGVAGSLVSGSCSQIINGEDCSPHSQPWQAALVMENELFC
+
PSA GGVLVHPQVLTAAHCKIRNKSVILLGRHSLFHPEDT-GQVFQVSHSFPHPLYDMSLLKNR
KLK2 GGVLVHPQVLTAAHCKLKNQSVWLGRHNLFPEDT-GQRVPVSHSFPHPLYNMSLLKHQ
KLK1 GGILVHRQVLTAAHCKISDNQVLGRHNLFDDENT-AQFVHVSESPHPGFMNMLLENH
trypsinogen GGSLINEQVVSAGCHYKSRIVQRLGEHNI EVLEGN-AQFVINAACKIRHPQYDRKTLNN-
KLK-L3 GATLISDRVLTAAHCKRKPVLVWRLGEHHLWKWEGP-EQLFRVTFDFPHPGFNKDLAN-
TLSP GATLIAIRVLTAAHCKLKPRIYVHLGOHNLQKEGG-EQTRTATESFPHPGFNNSLNNK-
neuropsin GGVLVGGNVLTAAHCKKKPKYTVRLGDHSLQNKDGP-EQBIIPVQSIHPHCYNSSD-VE-
zyme GGVVLHPLVLTAAHCKKPNLVQVLGKHNLRQRESS-SEQSSVVRVHIDPYDAAS----
HSCCE GGVLVNERNLTAAHCKMNEYTVHLGSDTLGDRR--AQRKAKASKSFRHPGYSTOT----
protease SGVLVHPQVLTAAHCKFQNSYTIIGLGLHSLBADQEPGSGMVESASLVRHPEYNRPLLAN-
+
PSA FLRPGDSSSDMLRLSEPAE-LTDAVKVMDLPTQEPALGTTTCYASGWGSIIEPEFLTP
KLK2 FLRPGDSSSDMLRLSEPAK-ITDVVKVLGLPTQEPALGTTTCYASGWGSIIEPEFLRP
KLK1 TRQADEYSDMLRLTLEPADTITDAVKVVELPTEEEVGSCTCLASGWGSIIEPENFSFP
trypsinogen -----DTMLTKLSSRAV-INARVSTISLTPATPATGKTCLISGWGNTASSGADVP
KLK-L3 -----DHNDTMLTRLRQAR-LSPAVQPLNLSQTCVSPGMCLISGWGAVSSPKALP
TLSP -----DHNDTMLTKMASPVS-ITMAVRPLTLLSRVCITAGTSCCLISGWGSTSSPOLRLP
neuropsin -----DHNDTMLTKLRLDQAS-LGSKVKPISLADHCTQPOQKCTVSGWGTVTSPRENF
zyme -----HDQDITMLRLRAPAK-LSELIQPLPLERDCSANTTSCSHLWGKTDAG--DFP
HSCCE -----HVDITMLTKLNSQAR-LSSMVKKVRLPSRCEPFGTCTVSGWGTTSPTDVTFP
protease -----DTMLTKLDESVS-ESDTIRSTISIASQCTPAGNSCLVSGWGLLANG--RMP
+
PSA KKLQCVDLHVISNDVCAQVHPQKVTFMKLCAGRWTGGKSTCSGDSGGELVNCNGVLGITS
KLK2 RSLQCVDLHLLSNDMCARAYSEKVTFFMLCAGLWTGGKDTCCGDSGGELVNCNGVLGITS
KLK1 DDLQCVDLKILPNDECKKAHVQKVTFFMLCVGLHEGGKDTCCGDSGGELMCDGVLGQVTS
trypsinogen DELQCLDAPVLQSAQCEASYPGKITSNMFCVGLFEGGKDSQCGDSGGELVNCNGVLGQVTS
KLK-L3 VTLQCANISILENKLCHWAYPGHISDSMLCAGLWEGGRGSCQCGDSGGELVNCNGVLGQVTS
TLSP HTLRCANITIIHQKCEKNAVPGNITDMVCAVQEGGKDSQCGDSGGELVNCNGVLGQVTS
neuropsin DTLNCAEVKIPQKKCEDAYPGQITDMGVCAGSSKG-ADTCCGDSGGELVNCNGVLGQVTS
zyme DTQCAIHLVSRREECHAYPGQITQNMKLCAGDEKYKGDSCQCGDSGGELVNCNGVLGQVTS
HSCCE SDLMCVDKLISPDQCTKVYKDLLENSMLCAGIDPSKKNACNCGDSGGELVNCNGVLGQVTS
protease TVLQCVNVSVEEVCCKLYDPLYPHSMFCAGGGHDKQDSCNCGDSGGELVNCNGVLGQVTS
+
PSA WGSEPCALPERPSLYTKVHVYRKWKIDTIVANP
KLK2 WGPEPCALPEKPAVYTKVHVYRKWKIDTIAANP
KLK1 WGVVPCGTGNPKPSVAVRVLSYVKWIEDTIAENS
trypsinogen WG-DGCAQKNKPGVYTKVYNYVKWKINTIAANS
KLK-L3 GGAEPSCSRPRPAVYTSVCHYLDWIQIEMEN--
TLSP WGQDPCAITRPGVYTKVYKYVDWIQETMKNN-
neuropsin WGSDDPCGRSDKPGVYTNICRYLDWIKKIIGSKG
zyme WGNIPGGSKEKPGVYTNVCRYTNWIKTIQAK-
HSCCE WGTPTCGQPNDPGVYTVQCKFTKWINDTMKHHR
protease FGKAPCGQGVPGVYTNLCKFTWIEKTVQAS-
+

```



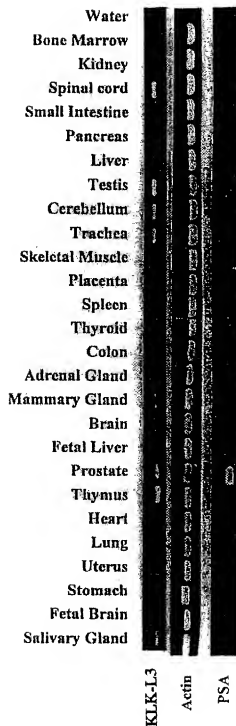
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## FIGURE 18

```

          +-----TLSP
        +---4
        !
        +-----KLK-L3
      +-13
        !
        ! +-----neuropsin
        ! +---12
      +-14 +-----NES 1
        !
        ! +-----zyme
        ! +---11
        ! +-----KLK-L4
        !
        !
        ! +-----PSA
        ! +---2
        ! +-----3 +---KLK2
        !
        ! +-----KLK1
      -15-10
        !
        ! +-----trypsinogen I
        !
        !
        ! +-----complement factor D
        ! +---9 +---6
        !
        ! +---7 +-----granzyme A
        !
        ! +---8 +-----cathepsin G
        !
        ! +-----chymotrypsin
        !
        !
        ! +-----protease/KLK-L1
        ! +-----1
      +---5 +EMSP
        !
        +-----KLK-L2

```

**FIGURE 19**

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## FIGURE 20

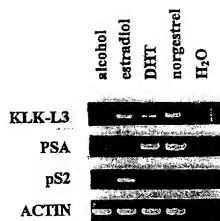


FIGURE 21

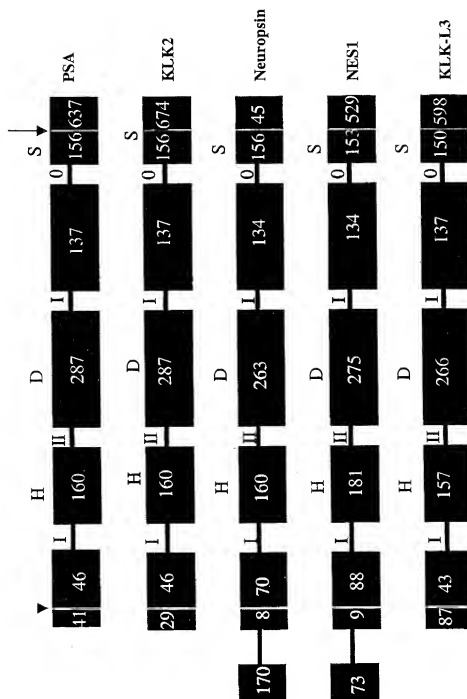
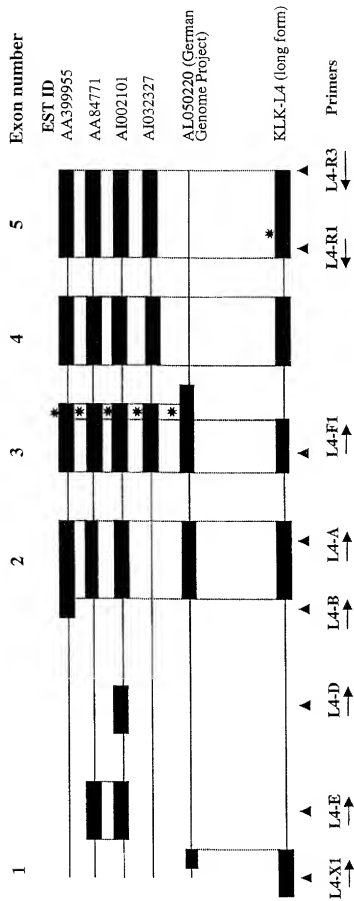
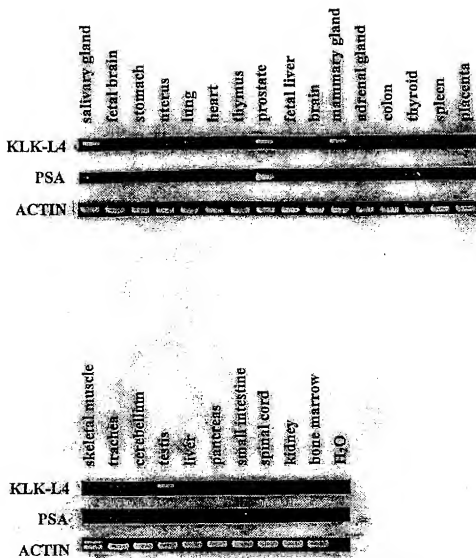


FIGURE 22

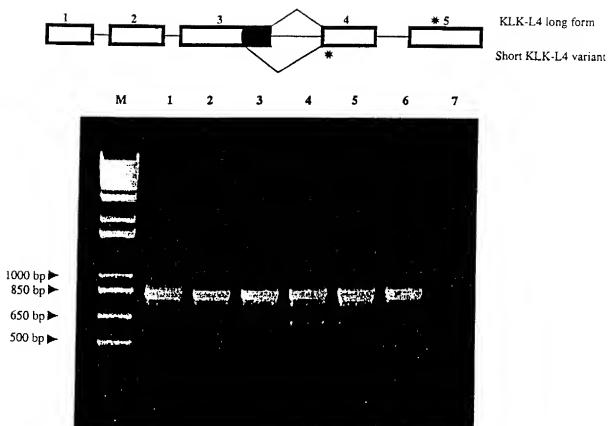


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FIGURE 23



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**FIGURE 24**

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FIGURE 25

TCAGGCCCCGCCGCCCTGCCCTCCCTCCCGATCCCGGAGGCC (ATG) TGG CCC CTG GCC  
M W P L A  
CTA GTG ATC GCC TCC CTG ACC TTG GCC TTG TCA GGA G...gtaaga.... intron 1 ..... ttaccag  
L V I A S L T L A L S G

GT GTC TCC CAG GAG TCT TCC AAG GTT CTC AAC ACC AAT GGG ACC AGT GGG TTT  
G V S Q E S S K V L N T N G T S G F

CTC CCA GGT GGC TAC ACC TGC TTC CCC CAC TCT CAG CCC TGG CAG GCT GCC  
L P G G Y T C F P H S Q P W Q A A

CTA CTA GTG CAA GGG CGG CTA CTC TGT GGG GGA GTC CTG GTC CAC CCC AAA  
L L V Q G R L L C G G V L V H P K

TGG GTC CTC ACT GCC GCA CAC TGT CTA AAG GA gtatgt ..... intron 2 ..... cacag G GGG  
W V L T A A [H] C L K E G

CTC AAA GTT TAC CTA GGC AAG CAC GCC CTA GGG CGT GTG GAA GCT GGT GAG  
L K V Y L G K H A L G R V E A G E

CAG GTG AGG GAA GTT GTC CAC TCT ATC CCC CAC CCT GAA TAC CGG AGA AGC  
Q V R E V V H S I P H P E Y R R S

CCC ACC CAC CTG AAC CAC GAC CAT GAC ATC ATG CTT CTG GTC CAG TCC  
P T H L N H D H [D] I M L L E L Q S

CCG GTC CAG CTC ACA GGC TAC ATC CAA ACC CTG CCC CTT TCC CAC AAC AAC CGC  
P V Q L T G Y I Q T L P L S H N N R

CTA ACC CCT GGC ACC ACC TGT CGG GTG TCT GGC TGG GGC ACC ACC ACC AGC  
L T P G T T C R V S G W G T T T S

CCC CAG G gatgcac.... intron 3..... atcc ag TG AAT TAC CCC AAA ACT CTA CAA TGT GCC  
P Q V N Y P K T L Q C A

AAC ATC CAA CTT CGC TCA GAT GAG GAG TGT CGT CAA GTC TAC CCA GGA AAG  
N I Q L R S D E E C R Q V Y P G K

ATC ACT GAC AAC ATG TTG TGT GCC GGC ACA AAA GAG GGT GGC AAA GAC TCC  
I T D N M L C A G T K E G G K D S

TGT GAG gatgca.... intron 4..... aactcag GGT GAC TCT GGG GGC CCC CTG GTC TGT AAC  
C E G D [S] G G P L V C N

AGA ACA CTG TAT GGC ATC GTC TCC TGG GGA GAC TTC CCA TGT GGG CAA CCT  
R T L Y G I V S W G D F P C G Q P

GAC CGG CCT GGT GTC TAC ACC CGT GTC TCA AGA TAC GTC CTG TGG ATC CGT  
D R P G V Y T R V S R Y V L W I R



## FIGURE 25 (CONT'D)

GAA ACA ATC CGA AAA TAT GAA ACC CAG CAG CAA AAA TGG TTG AAG GGC CCA  
E T I R K Y E T Q Q Q K W L K G P

CAA (TAA) AAGTTGAGAAATGTACCGGCTTCCATCCTGTCACCATGACTTCCTCAC  
Q

ATGGTCTGCTTAGCCCTTCTCTGCTCCTTATGCCAGTGTTCCATTTGAACCAGTGATCCATGTC  
CTGAAAAATGCTCAATCTCAGCTAACATTCCATGTTTCAGAAGCAITCAGGCACTGCCAGGCT  
TGCAGTCTCCCAGATGTTCATCCCTGAAACATCTCAACAACCTGAATGTCCCAACCCAGACA  
ATGGCCCAGGTCTCTCAACTTCATCAGTGTGGCTTCTATGAGCCCAGATCACCACTGAACGT  
TCTGTCTGTGGCACATTCTTAAATATTTCCATCAGCCCATCTCAACAATATATGTCCTATAAAAT  
GGACCATCCTTGACA

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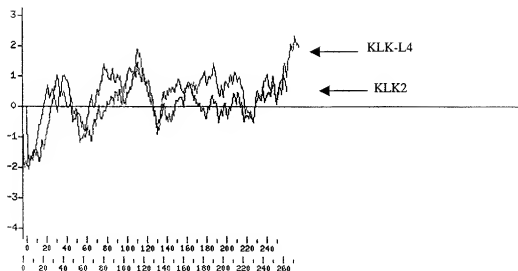
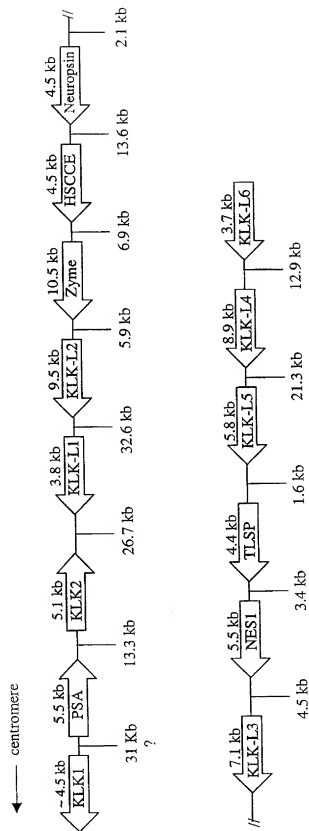
**FIGURE 26**

FIGURE 27

1	15 16	30 31	45 46	50 61	75 76	90
1	KLK-L1/protease	MRNGLG---VILG GAG	---	SLVSG	---	INERCPHSQPMQ
2	EMSP	MRNPG	---	SLVSG	---	INERCPHSQPMQ
3	KLK-L2	MRNPG	MRNPG	---	---	INERCPHSQPMQ
4	PSA	MRNPG	MRNPG	---	---	INERCPHSQPMQ
5	KLK1	MRNPG	MRNPG	---	---	INERCPHSQPMQ
6	KLK1	MRNPG	MRNPG	---	---	INERCPHSQPMQ
7	trypsinogen	MRNPG	MRNPG	---	---	INERCPHSQPMQ
8	zyme/protease M	MRNPG	MRNPG	---	---	INERCPHSQPMQ
9	KLK-L4	MRNPG	MRNPG	---	---	INERCPHSQPMQ
10	TLSP	MRNPG	MRNPG	---	---	INERCPHSQPMQ
11	neutropsin	MRNPG	MRNPG	---	---	INERCPHSQPMQ
12	NE51	MRNPG	MRNPG	---	---	INERCPHSQPMQ
1	protease	MRNPG	MRNPG	---	---	INERCPHSQPMQ
2	EMSP	MRNPG	MRNPG	---	---	INERCPHSQPMQ
3	KLK-L2	MRNPG	MRNPG	---	---	INERCPHSQPMQ
4	PSA	MRNPG	MRNPG	---	---	INERCPHSQPMQ
5	KLK1	MRNPG	MRNPG	---	---	INERCPHSQPMQ
6	KLK1	MRNPG	MRNPG	---	---	INERCPHSQPMQ
7	trypsinogen	MRNPG	MRNPG	---	---	INERCPHSQPMQ
8	zyme	MRNPG	MRNPG	---	---	INERCPHSQPMQ
9	KLK-L4	MRNPG	MRNPG	---	---	INERCPHSQPMQ
10	TLSP	MRNPG	MRNPG	---	---	INERCPHSQPMQ
11	neutropsin	MRNPG	MRNPG	---	---	INERCPHSQPMQ
12	NE51	MRNPG	MRNPG	---	---	INERCPHSQPMQ
1	protease	MRNPG	MRNPG	---	---	INERCPHSQPMQ
2	EMSP	MRNPG	MRNPG	---	---	INERCPHSQPMQ
3	KLK-L2	MRNPG	MRNPG	---	---	INERCPHSQPMQ
4	PSA	MRNPG	MRNPG	---	---	INERCPHSQPMQ
5	KLK1	MRNPG	MRNPG	---	---	INERCPHSQPMQ
6	KLK1	MRNPG	MRNPG	---	---	INERCPHSQPMQ
7	trypsinogen	MRNPG	MRNPG	---	---	INERCPHSQPMQ
8	zyme	MRNPG	MRNPG	---	---	INERCPHSQPMQ
9	KLK-L4	MRNPG	MRNPG	---	---	INERCPHSQPMQ
10	TLSP	MRNPG	MRNPG	---	---	INERCPHSQPMQ
11	neutropsin	MRNPG	MRNPG	---	---	INERCPHSQPMQ
12	NE51	MRNPG	MRNPG	---	---	INERCPHSQPMQ

## FIGURE 27 (CONT'D)

1	protease	271	285	286	300	301	315	316	330	331
2	ENB	SHLQGLVSG	KAPCGVGVGVYTN	LCKTWTMTQAS	-----	-----	-----	-----	-----	-----
3	KLK-L2	SHLQGLVSG	KAPCGVGVGVYTN	LCKTWTMTQAS	-----	-----	-----	-----	-----	-----
4	PSA	SHLQGLVSG	SPCATNRGVYTN	LCKTWTMTQAS	-----	-----	-----	-----	-----	-----
5	KLK2	SHLQGLVSG	SEPCALPKNVIR	VHTRGMKOTIVAN	P	-----	-----	-----	-----	-----
6	KLK1	SHLQGLVSG	SEPCALPKNVIR	VHTRGMKOTIVAN	P	-----	-----	-----	-----	-----
7	trypsinogen	SHLQGLVSG	VPCGTPKNSVIR	VHTRGMKOTIVAN	S	-----	-----	-----	-----	-----
8	zyme	SHLQGLVSG	DECAQENKNSVIR	VHTRGMKOTIVAN	S	-----	-----	-----	-----	-----
9	KLK-L4	SHLQGLVSG	NPCGSRHGVYTN	VHTRGMKOTIVAN	S	-----	-----	-----	-----	-----
10	TLSP	SHLQGLVSG	DFPCGQPIRGVYTN	VHTRGMKOTIVAN	S	-----	-----	-----	-----	-----
11	neuropilin	SHLQGLVSG	QDPCATRKGVYTN	VHTRGMKOTIVAN	S	-----	-----	-----	-----	-----
12	NEB1	SHLQGLVSG	SDPCGSRKGVYTN	ICRYLDMIKITGSK	G	-----	-----	-----	-----	-----

**FIGURE 28**

## FIGURE 29

```

+-----TLSP
+---4
! +-----KLK-L3
+---13
! ! +-----neuropsin
! +---12
+---14 +-----NES1
! !
! ! +-----zyme
! +---11
! +-----KLK-L4
!
! +-----PSA
! +---2
! +-----3 +---KLK2
! !
! ! +-----KLK1
-15-10
! ! +-----trypsinogen I
! ! !
! ! ! +-----complement factor D
! +---9 +---6
! ! +---7 +-----granzyme A
! ! !
! +---8 +-----cathepsin G
! !
! +-----chymotrypsin
!
! +-----prostase/KLK-L1
! +-----1
+---5 +-----EMSP
!
+-----KLK-L2

```

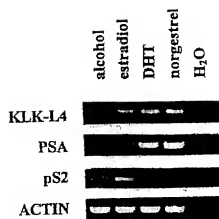
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**FIGURE 30**

FIGURE 31

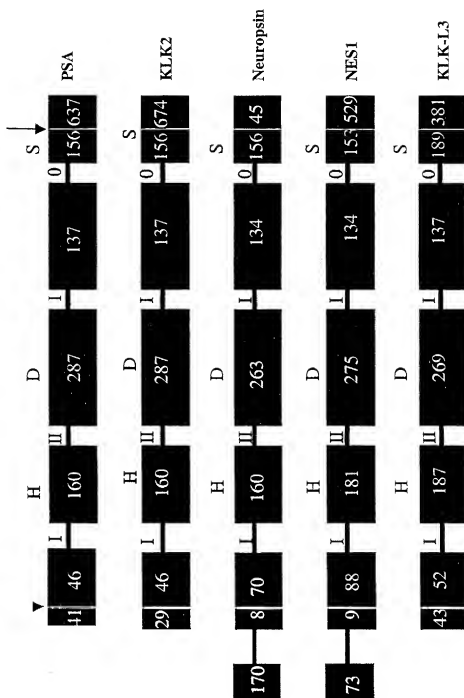
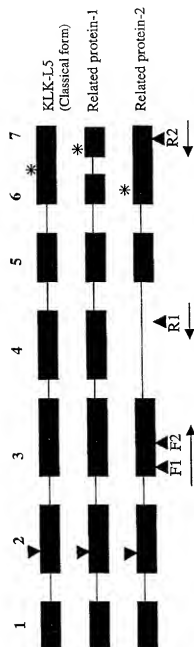




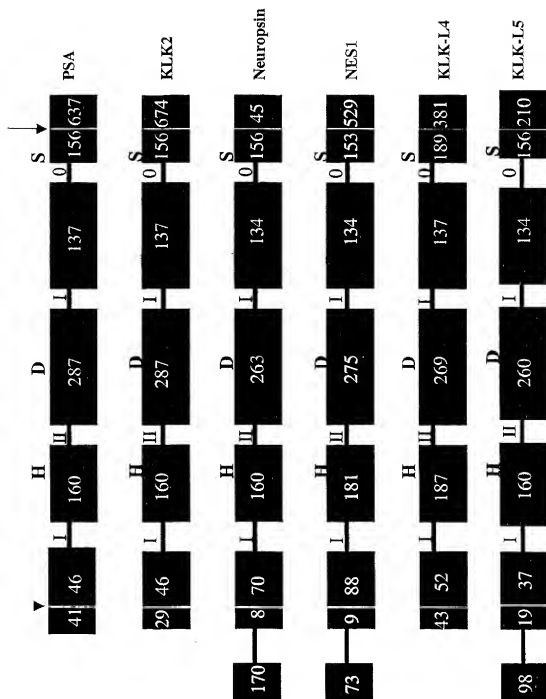
FIGURE 32



## FIGURE 33

GCAGGTAGGTGGACGGAGAGATAGCAGCGACGAGGACAGGCCAAACAGTGACAGCCACG  
 TAGAGGATCTGGCAGACAAAGAGACAAAGGTGAGAAGGAG gtagg.....Intron 1.....  
 .....tgacactccccag ACTTTGGAAGTGACCCACC(ATG)  
 M  
 GGG CTC AGC ATC TTT TTG CTC CTG TGT GTT CTT G gtagattctccg  
 G L S I F L L L C V L  
 gagcaggagagggca..... Intron 2 .....ctgtctgtctccag GG CTC  
 G L  
 AGC CAG GCA GCC ACA CCG AAG ATT TTC AAT GGC ACT GAG TGT GGG  
 S Q A A T P K I F N G T E C G  
 CGT AAC TCA CAG CCG TGG CAG GTG GGG CTG TTT GAG GGC ACC AGC  
 R N S Q P W Q V G L F E G T S  
 CTG CGC TGC GGG GGT GTC CTT ATT GAC CAC AGG TGG GTC CTC ACA  
 L R C G G V L I D H R W V L T  
 GCG GCT CAC TGC AGC GGC AG gtaagtccttcc.....intron3.....  
 A A H C S G S  
 .ccgtcgccaccggcag C AGG TAC TGG GTG CGC CTG GGG GAA CAC AGC  
 R Y W V R L G E H S  
 CTC AGC CAG CTC GAC TGG ACC GAG CAG ATC CGG CAC AGC GGC TTC  
 L S Q L D W T E Q I R H S G F  
 TCT GTG ACC CAT CCC GGC TAC CTG GGA GCC TCG ACG AGC CAC GAG  
 S V T H P G Y L G A S T S H E  
 CAC GAC CTC CGG CTG CTG CGG CTG CGC CTG CCC CTC CGC GTA ACC  
 H D L R L L R L R L P V R V T  
 AGC AGC GTT CAA CCC CTG CCC CTG CCC AAT GAC TGT GCA ACC GCT  
 S S V Q P L P L P N D C A T A  
 GGC ACC GAG TGC CAC GTC TCA GGC TGG GGC ATC ACC AAC CAC CCA  
 G T E C H V S G W G I T N H P  
 CCG A gtaaggggcccaggccagg.....intron 4 .....  
 R  
 .gaccctgcagcagcatgttctctctccag AC CCA TTC CCG GAT CTG CTC  
 N P F P D L L  
 CAG TGC CTC AAC CTC TCC ATC GTC TCC CAT GCC ACC TGC CAT GGT  
 Q C L N L S I V S H A T C H G  
 GTG TAT CCC GGG AGA ATC ACG AGC AAC ATG GTG TGT GCA GGC GGC  
 V Y P G R I T S N M V C A G G  
 GTC CCG GGG CAG GAT GCC TGC CAG gtagacc..... Intron 5 .....  
 V P G Q D A C Q  
 .aaaacagaataagatgtctccctgttcagacagtacttctcttccctccag GGT  
 G  
 GAT TCT GGG GGC CCC CTG GTG TGT GGG GGA GTC CTT CAA GGT CTG  
 D S G G P L V C G G V L Q G L  
 GTG TCC TGG GGG TCT GTG GGG CCC TGT GGA CAA GAT GGC ATC CCT  
 V S W G S V G P C G Q D G I P  
 GGA GTC TAC ACC TAT ATT TGC AA(G TAT GTG GAC TGG ATC CGG ATG  
 G V Y T Y I C K Y V D W I R M  
 ATC ATG AGG AAC AAC TGA CCGTTTCCCTCCACCTGCCCCACCCCTTATCTT  
 I M R N N  
 GGGTACCCCTCTGGCCCTCAGACACCAATATCTCTCCATCACTTCCCCCTAG) CTCCAC  
 TCTTGTGTGGCCTGGGAACCTTCTTGAACCTTTAACTCCTGCCAGCCCTTC(TAA) GACCCACG  
 AGCGGGGTGAGAGAAGTGTGCAATAGTCTGGAATAAATATAAATGAAGAGGGGGC

FIGURE 34



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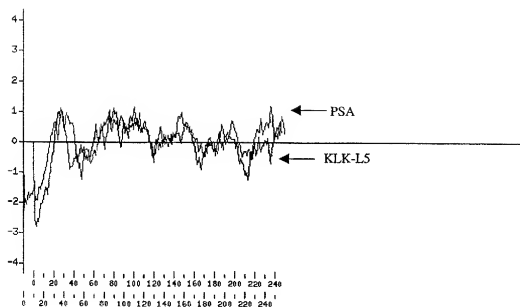
**FIGURE 35**

Figure 36

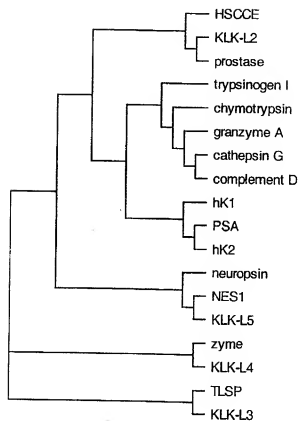
1	PSA	15 16	30 31	45 46	60 61	75 76	90
2	hK1	---	---	---	---	---	---
3	hK2	---	---	---	---	---	---
4	protease	---	---	---	---	---	---
5	zyme	---	---	---	---	---	---
6	TLSP	---	---	---	---	---	---
7	hK1-L4	---	---	---	---	---	---
8	hK1	---	---	---	---	---	---
9	hK1-L5	---	---	---	---	---	---
10	neuropilin	---	---	---	---	---	---
11	PSA	105 106	120 121	135 136	150 151	165 166	180
12	hK2	---	---	---	---	---	---
13	hK1	---	---	---	---	---	---
14	protease	---	---	---	---	---	---
15	zyme	---	---	---	---	---	---
16	TLSP	---	---	---	---	---	---
17	hK1-L4	---	---	---	---	---	---
18	hK1	---	---	---	---	---	---
19	hK1-L5	---	---	---	---	---	---
20	neuropilin	---	---	---	---	---	---
21	PSA	195 196	210 211	225 226	240 241	255 256	270
22	hK2	---	---	---	---	---	---
23	hK1	---	---	---	---	---	---
24	protease	---	---	---	---	---	---
25	zyme	---	---	---	---	---	---
26	TLSP	---	---	---	---	---	---
27	hK1-L4	---	---	---	---	---	---
28	hK1	---	---	---	---	---	---
29	hK1-L5	---	---	---	---	---	---
30	neuropilin	---	---	---	---	---	---

Figure 36 cont'd

		265	286	300	301	
1	PSA	SLAKKGVVKKKED	TIANP	---	---	261
2	HK2	AVTKGVVKKKED	TIANP	---	---	261
3	HK1	SVAVKLVKKKED	TIANP	---	---	262
4	protease	GVVNLKPTETLEK	TIANS	---	---	254
5	zyme	GVVNVKVTNLIK	TIQAK	---	---	244
6	TLSP	GVVNVKVTNLIK	TIQAK	---	---	250
7	KLK-L4	GVVKSRYVLNRE	TIKRVETQQCKKLG	PQ	---	277
8	NESL	AVTQICKVMSRIK	VIKRN	---	---	276
9	KLK-L5	GVVTVCKVVMIM	IKRNK	---	---	248
10	neutropsin	GVVTVCKVLMKK	IKSKG	---	---	260

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## FIGURE 37



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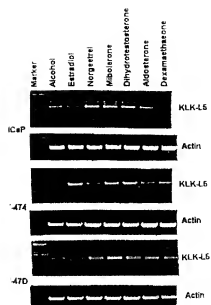
## FIGURE 38



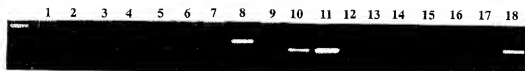


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## FIGURE 39



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**FIGURE 40**

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FIGURE 41

ATCGTGTAAAT	CACCGCCACA	TCCAGTGCAA	AGCTGATTCTG	TCACCACAGA	GCAGCTCCCT
CCTGCCACCC	CATCCCTCGG	TCCCAAGAGA	ACCCCTTCTT	AAAAGAGGGA	GTCTCTTGACG
GGTGTGGGG	GTCCATGCTT	TAATCTCTGC	ACTTTGGGAG	GCACAGGAGG	GTGGATCATTT
TGAGGTCAGG	AGTTTGCAGC	CAGACTGGCC	AACATGGTGA	AACCCCTGCTC	TTACTAAAAA
TACAAAAAAA	TGAGCGGGGC	ATGCTGGTGG	GTGCTTATAG	CCCCAGCTAC	TCAGGAGGCT
GAGGCAGGAG	AATCGCTTGA	ACCCAGGAGG	AGTAGGTTGC	AGTGAGCCGA	GATTGAGCCA
CTGCATCCCA	GCCCGGGGTA	AAGAGTGAGA	CTCTGTCTCA	AAAAAAGGGA	AAAGAAAAAG
AAAAAAGAA	AAAAAATAA	AATAAATAA	TAAATAAAAT	AAATTAAAAA	ATTAAAAAAT
AAAGAGGGGG	TTCTTGTGTT	GATGCCGAGC	CTGAACCAAG	GCAGAGGAGG	CCGGGAAGGC
TTCCCAAGGC	CTTCAGCTCA	AACGAGGAG	GCCCATAGTT	AAACGAAAC	AGTTTCAGGAA
TCACACAAAG	GCACCTGGGG	AGAGATGGGT	GTGTGCTCC	AGATGCAGGT	GCCCAGACAG
TGCGTCCCCA	GGGTGTACAGA	CAGACCCAGG	CCAAGCTCCA	GCTCAAGAG	CCAGCTTAGG
GGGTGTCCGA	GGTGGAGGGA	GGCTGAGTCA	GGCTGAGGCC	GGCGAACAGT	TGGGGTAGCC
AAGGGAGGCA	AGCAGCCTCC	TGAGTCACCA	CGTGTCCAG	GTACGGGGCT	CCCCAGCGCC
AGAGACGGAC	ACAAGCACTG	GGGAATTTAA	GGGGCTAGGG	GAGGGGCTGA	GAGGGGTATC
CCCTCCCCCA	AATGAGGATG	GAACCCCCC	AACCTCCAGAA	CCCCCTGCA	GGCTGGCCAG
AATCCTTCCC	CATCTCATTC	ACTCTGTCTC	TCTCTGTCTC	TGCCGTCTCC	TATTTTGAAT
TTCCAAACCC	GTCTGTAAAG	ACTGTCTCTC	TGCTCTGAA	TCTCTGTCCC	CTCTCTCTTC
TGGGTCTCTC	TCCCTCTCCC	TCTGGGTCTC	TGTCCCCCTC	TCTGGGTCTC	CTCATCTCTC
TCCTTTGCAT	TCAGCTCTCT	ACTTTGTCTC	<b>TGCACCTAGC</b>	<b>AGATCCCAAG</b>	<b>CTGGGGGAATG</b>
<b>CCAGTCTCTG</b>	<b>CACCAACCTT</b>	<b>CCTGCTCCCT</b>	<b>GCTGGGGCCT</b>	<b>CTGCTCCCCC</b>	<b>ATCTCTCAGG</b>
<b>AGTCGAAAGT</b>	<b>GAGAAAGCAA</b>	<b>GGTGGGCAGC</b>	<b>TCTGCTCCAG</b>	<b>GTCCAGGTTAT</b>	<b>CTCCCGCCCA</b>
CCTCCTGCCC	GTCTCTATC	CCACCCCTC	TCTCCATCTC	TCCCTTGGCG	TCCCATCTCT
CATCTAGGCC	TCCGCTCTCT	CTGTCAATGT	CCCCATCCCC	TGTAGGTGCT	CATCCTCTCC
CTCTTCCCCC	TGCCATCGGC	CTGCTGTGCC	CATCCTCTTT	GTCCACCACT	GTCCCGTTCT
CTTCCACGTC	TGATGCCCGC	ACTGCCCTCA	TCATCATCGC	TGTTTGTCTG	TGTGTGTTTG
TGTTGAGTGC	CGCATGGTGG	GGCGCTCTCG	CGCTCTCTCC	TCTCTCTCCA	CTGT'TTTCTC
TTTCTGTGTG	TCGTGTTCCA	TTCTATCTCC	ACCTTCTCTC	CTCGGTCTTT	TGCTT'TTCTA
TCTCCACTTC	TCCACACCCC	TCTCTCCCTG	CGTCTCTGTG	TCTCCCTCTC	CCTCTGTCTT
GT'TTT'TTCC	CACCGTCTGC	CTCTCTCTGT	CCCTGTCAAC	TCCAACCTTC	ACCGGTTTCT
CCAGCTCTCT	CCTCAGTTCC	TTCTCTCATG	AGCACACCTG	CTCTCTGTCT	CGTATTCCTG
GACTCCTCTC	TCTCCACTGT	CATATCTTCT	CATCTATTTT	CCCAGTCTCT	CTCTGTCTCT
TGCTCTCCCC	CTCTCTGTCA	CTCTGTCTCT	GTCTCTCTCT	TTCTCTCTCT	CTCTCTGTGT
CTCTCTGTCT	GGCTCTCTCT	CTGTCTCTCT	CTCCATCTCT	CTCTCTCTCT	CCCCCCCGTC
ACCTCTGTCT	TGCTCTCTCT	TGCTGTGTGT	TTCTCTCTGT	TTTCTCTCTC	TCCATCTCTC
TCTGTCTCTC	TCTCTCTCTC	TCTCTCTCTC	CTCTCTCTCC	TCCCTCCGCT	ACTCCCTCTC
TCAGTCCATC	TCTTCTCTCC	TCTCTCAGCC	CCTTCTGTGC	CTTCTCTCTG	ACACTCTCCA
CCCTGGTTTC	CTGACTCCAC	CACCTAGATCC	ACCACCTCCA	GCAACTGGGA	ACCTTCCCCC
GCCCACCCCT	CCCTGGGGTC	CCCTCCCAGG	ATTCTTCTTA	GATTATAGCA	CTCTCCCTGT
CGGGGTCTCT	ATGAGCAATT	GTGGCTGCTT	TTT'TGGCCAG	CAGAGGGGAG	GAGGGGATGG
TGACAGGAG	TCTCGGAATG	GGAACTAGGC	AATAAAAAAA	AAAAAATGTC	AGAAGCAGGG
CGCGGGGAG	TGGGGGCGAG	GCCAGCTGTC	CTTACCAGGG	ATAAAAGGCT	TTGCCAGTGT
GACTAGGAGA	AGAGACACCT	CCCCCTCTTC	CTTCATCAAG	<b>ACATCAAGGA</b>	<b>GGGACCTGTG</b>
<b>CCCTGCTCCA</b>	<b>CATCTCCCCA</b>	<b>CTGCGGCCCC</b>	<b>GCAGAGCCTG</b>	<b>CAGGCCCCGC</b>	<b>CCCCCTCGTC</b>
<b>TCGTGCTCCT</b>	<b>ACCTCTCTGC</b>	<b>TGTGCTCTTA</b>	<b>TGTCCCTGAG</b>	<b>GGTCTTGGGC</b>	<b>TCGTGTAAG</b>
TGCCCCCTGC	TGCTCTCTGC	TCTCAGCCCC	CGGTCTCTGT	GAAGGTCTCT	TCTCTCTCAT
TTT'TTCTCTG	CATTTTGACAG	<b>GACCTGGCCC</b>	<b>TCAGCCCCCTA</b>	<b>AAATGTCTCT</b>	<b>CTCTGTGACA</b>
<b>GCACTTCAAG</b>	<b>TCCTGGCTAT</b>	<b>AGTAAAGAGA</b>	<b>ACGGTTGGGT</b>	<b>ATGACACAAG</b>	<b>GGGGTCCCTT</b>
GGAGACTCTG	AGAAAGATG	GGGATGGGTC	CTTGGGGGCC	CTGGATGCTC	ATGGTGACCT
CATAAGAAAG	AGCAGGGAGT	GGTTTGGGGG	TCATGGTGGG	GGAACGTGGG	GAGAGCCCTAA
ATTCCTAGTT	GTGGAGGTGC	TAGGGAATTT	TGGGGCCGGG	GAGGAGGGTG	TTTATAAGGT
CTGGTGCAAA	ATACATAAGG	AATCTTAGGG	AACATATTAG	TCTGAGGTGC	GTCAATAGCAG
AAAGATACAG	GGGCTCTACC	TGACTGTGTT	AGGAAAGAAA	CAATGTCTAGA	AAGATGTTTT
GTGTCTCAG	GGAAGGTGGA	GAAGGATGAT	GGGATGGCGG	GATCTGGACA	TGGGGTGGCC
GGATCGTGGC	ATGSGTGTGT	GAGGTGGATG	GGGGCAAGTG	TGGGGCAAG	GATGGCCGAT
CCTTGGGGTG	CCACTGAGTG	GGAACGTTGG	GGAGGAGACA	GGGAGGTCTC	TGAATGTGTT
GGGGAGAGAC	TCATTGGGGG	GAAATGTGGC	ATATTTCGAG	AAGTGTGACG	GAAATATATG
GGAGCATAGA	GCTAAGGGTC	GTAGATGTAG	CAAGGCCCTG	GATAAGGTGG	CCACGGACA
AAATAAGAGA	TGCTACGGAG	GTGACTTGGG	AGGTGAGTCA	GAAAGCTCTC	CGTCTGGGG

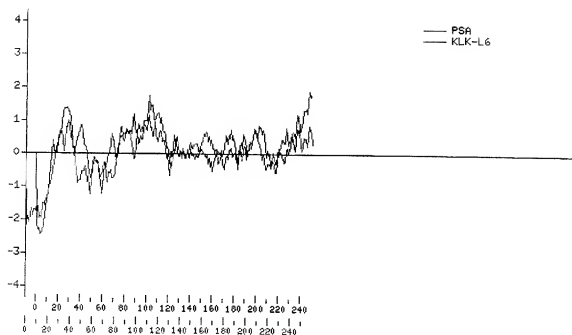
## FIGURE 41 (CONT'D)

CAATAACGGG GTCAATATTG GGCATGTCTC ACCCTGGGTG GGACAGATAG AGGCGGGCAG  
 TTATGGGGTT AGACCAAAAG GAAGGGGATT TGTCAGTTTT GGAATCTCTAC AAACCTTGTTG  
 AGTGGAGAGT GTTGTCTCAT CTACTTTCCC CACCCAATCC TGTCCTACTCC TAGCCATGAC  
ACAGAGCCAA GAGGATGAGA ACAAGATAAT TGGTGGCCAT ACGTGTCACCC GGAGCTCCCA  
GCCGTGGCAG GCGGCGCTGC TGGCGGGTCC CAGCGCGCCG TCTCTCGGCG GAGGCGCCCT  
GCTTTCAGCG CAGTGGGTCA TCACTGTCTG TCACTGGGCG CGCCCGTAAG TGACCCCTCT  
 CCCTGTCCCT GTACCTAGTG AATTCCAGAG TCTAAAGCCC TAGAGCTGAG CTGAGAACCT  
 GGATCTCTGT ATAGAACCAC ATGTAGTGGC TGGCTCTCGG TTTGAGGTCT AGAGAAGAGC  
 CTGGAACAAA AACACAGCTC GGGATGTGGG CTCTCCATCA AATCTCGAAC TCAGCATAGG  
 TTCTGAAAGC AGATGGGCAG CTTGGAACCC ATGGACCTGC TGAGAACCCA ACATCTGATG  
 CAGTGATTCT TCCAGAGGCC ACACATTACA TCGAGACCAA GCTTAGCCCA TTCCAGATTG  
 GTGGCTGAAT TCAGGACCCC GTCTACATTC AGAAACTCAG GACACTCAGT AGAACTCAGA  
 GCCCAGTTCA GGACCTGCAG TCTAGCCATA AATCCAGAAC TAGAACGCTG CTCACAGCTG  
 GAACATACAA CTCTAAGAAT AGAGGCCAAA CCTGGAGGCT GTTTCACACC CAAGGTTTAG  
 TTCAGAGTCT AGTCTATAGC TCCGCTATGA CGACACTTCA ACCCAGTGTT TGAATCCCG  
 AATGTGGCGG GTGCGGTGGC TCATGCCATAT AATCCTAGCA CTTTGGGATG CTCGAGCTAG  
 CAGATCACTT GAGGTCCAGGA GTTCGAGACC AGCCTGAGCA ACATAGAGAA ACATGTTCTC  
 TACTAAAAAT GCAAAAATTAG CCAGGCGATGG TGGCACATGC CTGTAATCCC AGCCACTCGG  
 GAGGCTGAGG CAGGAGAATC ACTTGAACCT GGGAGGCGGA GGTGTCAGTG AGTCAAGATC  
 GCACCATGTC ACTCCAGGCT AGGCCAACAG AGGCCAACTC CATATCAACT AATCAATCAA  
 TAAATCCCGC AATGCAGATC CTAATCAGAA GCCCCATATA AAACCTAGAC CCCTCCTAAA  
 TTCTAGATCT GAACCTACAA CCCAGACCCC AGCCAAAGG TCAAAATGCC TATAGCCAT  
 ATCTATGCCA TAAACAGGTC AGTCTAGAAC CTAGAGATCA AAGCTCAGGC CAGAGCTAG  
 AATATAAGG CCAGAATGCA AACCAGACTC TAGAATCTTG GATCCGGGCC ATAACCTAGA  
 GCTCCAACCTA GAACCCAGAG CCCAACCTGA GGTCAAGGGC TAGGGGCCAGA GTCAGAACCC  
 AAGAGCCCTA TAATCCAATA TGAACACAGC GTGTAGAGGC TGGGTGCGGT GGCTCAGCCG  
 TGTAAATCCA GCACCTTGGG AGGCTGAGGC GGGAGAAATCA TTGAACTCGG GAGTTGAGG  
 CTGAGAGTGA GCTGAGATCG TCCCACGTCA CTCACGCCA GGTGACAGAG CGAGACTTCA  
 TCACAAAAAA AAAATAAATA AATAAATCAA GTCAATACT AGGTTTCGAT TAGAATCCTG  
 ATCTTAGCAT AGAGTCAAAA GTTAAAGATG TCTAGAATCT AGAACCAGG CTAGAAACAG  
 AATGGTGCCT ACTCCGGAAT ATCAGTTCG ATTTAGAGCC TAGACTCTA ACAGCATTTT  
 GCTTAGGACT CAATGCACCG AGCCCAGCAC AGACCTTGGC ACGGAGCCAA GCTCTCCCAA  
 TCATCACTTT CTTCCCAAGC CAGGAGCTGG AGCCCAGCCC AAGAGCGGAA GGAGAGGCAG  
 CTGGGGCTGG GCCGAGAGAA TGCCCTGGCC ATGGGGAAGG GCACAGGAGG CCAAGATGCT  
 TCGGCCGTGA GTTAGTGAGA AGCAGGCTAG ACCTCGGGGA AGACTCGTCA CCCGGCCAGG  
 GAACCGGCTG GGAGGGTGGG GAGGAGTCTC TGGCTCAGAC CCTGAGCAGC GCTTCTCTTG  
 GGGGTCTGTG CACAGGATCT TCAGGTTGCC CTGGGCAAGC ACAACCTGAG GAGGCTGGAG  
GCCACCCAGC AGGTGCTGCG CGTGGTTCTG CAGGTGACGC ACCCCAATA CAATCCCGG  
ACCCACGACA ACCACCTCAT GCTGCTCGAG CTACAGCAGC CCGCAGGGAT CCGGAGGCCA  
GTGAGGCCCA CTAGAGTTCAC CCAGGCTGTG GCCAGGCCCG GGACCTCTCT CCGAGTGTCA  
GGCTGGGGAA CTATATCCAG CCCATCGGT GAGGACTCCT CGCTCTTGA AATCAGGGGA  
 CTGGGCCCTG GCTCTGGGT CTCCAGGAGG TGGAGCTGGG GGGACTTGGG CTTCTGGGTC  
 TGAGGGGCTGA GGGGCTGGGC CTGGACTCCT GGGTCTGAGG GAGGAGGGGG CTGAGGCCCTG  
 GACTCTGGG TCTCAAGGAG GAGGAGCTGG GCCTGGACTC ATACGTCTGA GGGAGGAGGG  
 CTGGAGGCTT GGACTCTTGG GTCTCAAGGA GGAGGGGGCT GGCCTGGACT TCTGGGTCTG  
 AGGAGGAGGG GGGTGGGGAC CTGGACTCCC GGGTCTGAG GAGGAGGGAC TGGGGGTCTG  
 GACTCTTGGG TCTGAGGGAG GAGGGGCTGG GGGGCTGGAC TCTGGGTCTG GAGGAGGAG  
 GTGCTGGGGG TGGACTCTGT GGTGGAAGG AGGAGGGGCT GGGGGGCTGG ACCTCTGGGT  
 CTTATGGGAG GGTAGACCCA GTTATAACCC TGCAGTGTCT CCCAGCCAGG TATCCCGGCT  
CTCTGCAATC CGTGAACATC AACATCTCCC CGGATGAGGT GTGCCAGAGG GCCTATCTTA  
GAGGACTCAC GCTTGGCATG GTCTGTGCGAG GAGTTCGCCA GGGCGGGAG GAGTCTCTGT  
 AGGTAAAGGCC CAGGATGGGA GCTGTGCTAG GGATATTATT GGAATGGGAT TTAAGCAAA  
 GATGTCAGGA GCATGGAAGT CTGCAGAGGT CTTCAGAAAG GAGTGAACCG CAGGACAGCA  
 GAGATTCCGA TAGCCAGGCC ACCCTGCTCT CTAGCCCTGG GCCCTCTGGG TAATGAGCTC  
 AGAGCATTC A TGCCTCAGTT TCCTCATCTG TCAGGTGGGA GTAAACCTCT TAGGGTAGTT  
 GGTGGAATGG GATGAGGCAG GTTGGGGAAA GATCGCAGAG TGGCCTCTCG TCATATGGGT

## FIGURE 41 (CONT'D)

CTGGGAAAGG	CTGTGCTGAG	GCTTCTAGAA	ATCTTAATGC	ATCCTTGAGG	GAGGCAGAGA
TGGGGAATA	GAAAAAGAGA	GACACACAAA	TGT'TCTACAG	TTGGAGCGAA	CAGAGAGGGG
CCTGGTGAGA	TTCAAGGGAC	AGGCAGGTGC	ACACAGAGAC	AGAGCCAGAC	CCAGCGGAGA
GGGAAGGAAG	TGCCCCGACC	TCCGGGGCTG	AGACCTCAGA	GCTGGGGCAG	GACT'GTGTCC
CTAATCTGTC	ACCGACTGTCT	CT'GCCCTGTCT	CCCTTGCTGT	GCT'TCTCGGG	TTCTCTGTGC
CATGCTGGCT	CT'GGCTACCT	GTCCATCAGT	GTCTCCATTT	CTGT'TCCTCC	CCC'ACAGGGT
<b>GACTCTGGGG</b>	<b>GACCCCTGGT</b>	<b>GTGCAGAGGA</b>	<b>CAGCTCCAGG</b>	<b>GCCTCGGTGC</b>	<b>TTGGGGGAATG</b>
<b>GAGCGCTGCG</b>	<b>CGCTCCCTGG</b>	<b>CTACCCCGGT</b>	<b>GTCTACACCA</b>	<b>ACCTGTGCGAA</b>	<b>GTGCAGAAAG</b>
<b>TGGATTGAGG</b>	<b>AAACGATGCG</b>	<b>GGACAAATGA</b>	<b>TGGTCTTAC</b>	<b>GGTGGGATGG</b>	<b>ACCTCGTCAG</b>
<b>TGCCCCAGCG</b>	<b>CGCTCTCTCT</b>	<b>CTACTCAGGA</b>	<b>CCCAGGAGTC</b>	<b>CAGGCCCCAG</b>	<b>CCCTCTCTCC</b>
<b>CTCAGACCCA</b>	<b>GAGATCCAGG</b>	<b>CCCCCAGCCC</b>	<b>CTCTCTCCCT</b>	<b>AGACCCGGGA</b>	<b>GTCCACGGCC</b>
<b>CACGCCCTCT</b>	<b>CTCCCTCAGA</b>	<b>CCCAGGAGTC</b>	<b>CAGGCCCCAG</b>	<b>CCCCCTCTCT</b>	<b>CTCAGACCCG</b>
<b>GGAGTCCAGG</b>	<b>CCCCCAGCCC</b>	<b>CTCTCTCCCT</b>	<b>AGACCCAGGA</b>	<b>GTCCAGGCCG</b>	<b>CAGTCCCTCC</b>
<b>TCCCTCAGAC</b>	<b>CCAGGAGTCC</b>	<b>AGGCCCCGAG</b>	<b>CCCCCTCTCT</b>	<b>CTCAGACCCA</b>	<b>GGATCCAGG</b>
<b>CCCAGCCCTCT</b>	<b>CTCTCCCTCAG</b>	<b>ACCCAGGAGC</b>	<b>CCCAGTCCCC</b>	<b>CAGCCCCCTC</b>	<b>TCCTTGAGAC</b>
<b>CCAGGAGTCC</b>	<b>AGGCCCAGCC</b>	<b>CTCTCTCCCC</b>	<b>CAGACCCAGG</b>	<b>AGCCCCAGTC</b>	<b>CCCAGCATCC</b>
<b>TGATCTTTTAC</b>	<b>TCCGGCTCTG</b>	<b>ATCTCTCCTT</b>	<b>TCCCAGAGCA</b>	<b>GTGCTTTCAG</b>	<b>GGCTTTTCTC</b>
<b>CCCACCAAGC</b>	<b>CCCCACCCCT</b>	<b>GCTGTGTAC</b>	<b>CATCACTACT</b>	<b>CAAGACCCGA</b>	<b>GGCACAGAGG</b>
<b>CAGGAGGCAC</b>	<b>AGACCCCTTA</b>	<b>AACCGGCATT</b>	<b>GTATTCCAAA</b>	<b>GACGACAAAT</b>	<b>TTTAAACACG</b>
<b>TTAGTGTCTC</b>	<b>TAAAAACCGA</b>	<b>ATAAATAATG</b>	<b>ACAATAAAAA</b>	<b>TGGAATCATC</b>	<b>CTAAATTTGA</b>
<b>TTCAATTCATC</b>	<b>CATGTGTTTA</b>	<b>CTTTTTATTT</b>	<b>TTTGAGACAA</b>	<b>GGTCTTGCCT</b>	<b>AGTCTCCTGG</b>
<b>TGAAATGCTG</b>	<b>TAACGCAATC</b>	<b>ATAGCTCACT</b>	<b>GCAACCGTGA</b>	<b>CCTCCTGGGG</b>	<b>TCCAGTGATC</b>
<b>CTCTTACCTC</b>	<b>AGGCTCCCGA</b>	<b>GTAGCTGGGA</b>	<b>CCACAGSTGC</b>	<b>CCGTCAACAT</b>	<b>CGCCCGCTAC</b>
<b>TTT'TTAAAT</b>	<b>TTGTGTAGAG</b>	<b>ATGAGGTTTC</b>	<b>CCGTGTGTGC</b>	<b>TCAGGCTGGT</b>	<b>CTCGAACACC</b>
<b>TGACCCCAAG</b>	<b>CAATCCGCTC</b>	<b>ACGTCCGGTTT</b>	<b>CCCAAGTGTG</b>	<b>CGGAGTTGCA</b>	<b>GGCTGTGAGT</b>
<b>GCCGCCGCCA</b>	<b>GCCTTATCCA</b>	<b>TCGAATTAAT</b>	<b>GACTTCAAGA</b>	<b>AACATGTACA</b>	<b>CAGTGGCCCC</b>
<b>ACCATGTCCAA</b>	<b>GCCAGGAGCT</b>	<b>GTGTACTGAC</b>	<b>AAGTGGCTGC</b>	<b>CTCCCTCTTT</b>	<b>GGGTGTTTTT</b>
<b>CCTTGGGAGT</b>	<b>CCCCCGTCCA</b>	<b>CCCCACTGTA</b>	<b>TCAGGTTTCT</b>	<b>AGACGGAAAC</b>	<b>ACCTCAGCCC</b>
<b>TGCAGAGTGA</b>	<b>CCTTGAGCAT</b>	<b>GACTGCCTTC</b>	<b>TACCAGCCTC</b>	<b>CTCCCTGGAG</b>	<b>CCCCGTGTGT</b>
<b>CCAGGGTAGG</b>	<b>GAACATAAGTG</b>	<b>CCTTGTTTCC</b>	<b>TGGAATAATG</b>	<b>TATGCAATAT</b>	<b>AAGATGTCTCT</b>
<b>CATT'TTCCFA</b>	<b>ATCAGATCTC</b>	<b>AGGTGAGGAG</b>	<b>AGT'TGAGTTA</b>	<b>ATCACAGGCT</b>	<b>TCAGTTCCTTG</b>
<b>CCCAGGCAAA</b>	<b>GCCCTTCTCT</b>	<b>CATT'TTATTA</b>	<b>ATTATTTTCC</b>	<b>ACTCTTCATC</b>	<b>TCGCTCTCTG</b>
<b>CTCCCCCTCC</b>	<b>TCCCCACAGG</b>	<b>CACCGACATA</b>	<b>AATGGCTTTG</b>	<b>AGTGCCTTGC</b>	<b>ATCTCTTGAA</b>
<b>AACAATGCCAG</b>	<b>TGTCACAGTG</b>	<b>TACTGTTTCT</b>	<b>AAITTTACATG</b>	<b>AAACCATTTG</b>	<b>GTTAGGAATC</b>
<b>TCAT'TCTCTT</b>	<b>TCTTACTTTC</b>	<b>ACTCATCAAC</b>	<b>AGCTAT'TGAG</b>	<b>CACCTACTAC</b>	<b>GGGCCAGGCA</b>
<b>TTGGTCTTAT</b>	<b>TATTAGGCAC</b>	<b>CTGCTATACA</b>	<b>CCAGGCATTG</b>	<b>TTCTGGGTGC</b>	<b>TGGAGGAAGA</b>
<b>ACTGTGAGCA</b>	<b>AGCCAGTCAG</b>	<b>AATCCCTGCC</b>	<b>CTCACAGAAC</b>	<b>TTATATTCTA</b>	<b>CGAGGAGATG</b>
<b>ACAGACAAGA</b>	<b>AGCCATAAAC</b>	<b>ATAATTTTAA</b>	<b>AATAAAGCAG</b>	<b>AGTCCC'TATG</b>	<b>AGTAACGAGG</b>
<b>TCAATAAACT</b>	<b>TGGGCTGGGC</b>	<b>GGCAGGCCCA</b>	<b>ATGTGTGCCA</b>	<b>GGGCCAGCTC</b>	<b>ATACATGCTC</b>
<b>GCAAGAGTCT</b>	<b>ACCAGCAAAAT</b>	<b>TTTCAGGAAT</b>	<b>TTGAGAACCC</b>	<b>AGTTCGTAAA</b>	<b>TGTCAGCCATC</b>
<b>ATTAATAAAT</b>	<b>AAAT'TACATA</b>	<b>AGCGTATAAT</b>	<b>TACATAATTG</b>	<b>ATTAATAAAT</b>	<b>TTGCTCAGTAA</b>
<b>TGCTTGGATA</b>	<b>CTCAACTGTT</b>	<b>GCTAATTAAT</b>	<b>TCAACTTAAT</b>	<b>CCATGCTGTG</b>	<b>GGAGTGAGAT</b>
<b>ATGTCTCTTG</b>	<b>TACTAGCTCT</b>	<b>GTAATGATGA</b>	<b>GT'TTCTGCAC</b>	<b>ACCTCTTTCC</b>	<b>AAC'TCCCCAA</b>
<b>CTCTGTCTGC</b>	<b>ACCAGTAGCT</b>	<b>TGACAATAGC</b>	<b>CAAGAAGAA</b>	<b>GTATTTACTG</b>	<b>CAGTGAATTT</b>
<b>GAAAAACACT</b>	<b>ATAGATAGGG</b>	<b>CTTTGGCCGA</b>	<b>CAGTCAATTGC</b>	<b>TAAACCTTTA</b>	<b>CCAGGCCACC</b>
<b>TTGGATGGGT</b>	<b>CTGCC'TGGGA</b>	<b>ATGACCTCAT</b>	<b>GATCTTAGTG</b>	<b>TCTGTCTTCT</b>	<b>CAAGT'TCTG</b>
<b>TGCTTGGATA</b>	<b>CTGCAGAGTA</b>	<b>TAGCTAAAAAT</b>	<b>AGAATGTTGT</b>	<b>ACTCACCTTA</b>	<b>TGTCTTATGG</b>
<b>GGACAGCACA</b>	<b>GTA'TTGGGGA</b>	<b>ACCCTAAGGT</b>	<b>GGCAGGCTG</b>	<b>GGACATGCAC</b>	<b>GAAAGATTGC</b>
<b>TGGGAAGTAG</b>	<b>AGGCTCCCTC</b>	<b>CTTTTCTCTA</b>	<b>TCTCTCCACC</b>	<b>CCATCTTCCA</b>	<b>GTGTCTGGTA</b>
<b>ACCACCATTC</b>	<b>TACTCTCTGC</b>	<b>TTCTAAGAGT</b>	<b>CTGAGTTTTT</b>	<b>TAGATT'TCAC</b>	<b>ATGTAAGTGA</b>
<b>GATCATTCAG</b>	<b>TAAT'TGTCAT</b>	<b>TCTGTGTCTG</b>	<b>ACCT'ATTTCA</b>	<b>CTTAACACAG</b>	<b>TGTCCTCCG</b>
<b>GTCCATCCAT</b>	<b>GTTGTACAAA</b>	<b>ATGACAGGAT</b>	<b>TTCTTCTTCT</b>	<b>TATAAGGCAG</b>	<b>AAT'AAATATG</b>
<b>AAT'TATACATG</b>	<b>ATACTAATAT</b>	<b>ATTACATTTT</b>	<b>CTT'TATCCAT</b>	<b>TCATCCATCA</b>	<b>ACAGACACAT</b>

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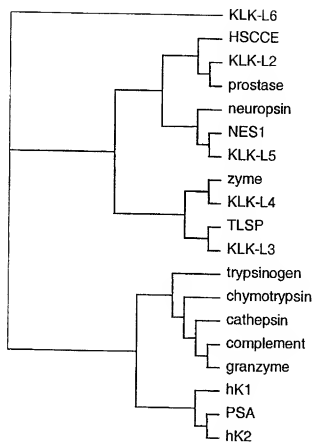
**FIGURE 42**

1	15	16	30	31	45	46	60	61	75	76	90
1	PSA	-----	MMVFVFLTLGV	TWIGAAPLLIS	-----	RIVGWMBCE	HSQSPQWLVASRG	-----	RAVCGVLVHPQWV	60	
2	hK2	-----	MDVLVSTALSV	GCTGAVPLIQS	-----	RIVGWMBCE	HSQSPQWLVASRG	-----	WAHCGVLVHPQWV	60	
3	hK1	-----	MMFVLVCLALS	GCTGAAPTIQS	-----	RIVGWMBCE	HSQSPQWLVASRG	-----	TFQCGGIIIVHPQWV	60	
4	HSCCE	-M	ASLLSLPLQLLSL	ALETGAPEAQGS	-----	RIVGWMBCE	HSQSPQWLVASRG	-----	QLHCGGVILHPLWV	65	
5	5yme	-----	MMKLMMVLSILAI	AMWAEQ	-----	NKIIHGGFPCD	TSHPYQAAIVTSG	-----	HLHCGGVILHPLWV	57	
6	6 K1K-16	-----	MMFLITLALQVIAI	AMTQSQDE	-----	NKIIHGGTCT	RSQSPQWQAALLAGR	-----	RPLFCGGLAGSGQWV	62	
7	7 TLSP	-----	MMFLQVILILALAT	ALVGGEYR	-----	LIIGFECK	HSQSPQWQAALLPEFT	-----	RLLCGATVLIAPKVL	57	
8	8 K1K-14	-----	MMFLQVILIASLT	ALVGGEYR	-----	LIIGFECK	HSQSPQWQAALLPEFT	-----	RLLCGATVLIAPKVL	57	
9	9 NES1	-----	MMFLQVILILALQVIA	AEALLIPQDTR-I	-----	DFEAVGAPCA	RSQSPQWQVSVFNGE	-----	SFHCAGVLVLDQSGWV	71	
10	10 K1K-15	-----	MMFLSTELLVLQVL	QAAPTRFYN	-----	DFEAVGAPCA	RSQSPQWQVSVFNGE	-----	SLRCGGLVLDHRRWV	57	
11	neuropsin	-----	PRAAKTMFELLQGL	QAAPTRFYN	-----	DFEAVGAPCA	RSQSPQWQVSVFNGE	-----	SLRCGGLVLDHRRWV	57	
12	protease	-----	MMFAGN	FWMGVITLIGLVAG	SVLSGSCS	-----	QINGSDCS	PHSQPQWQAALVME	-----	ELFCSGVLVHPQWV	66
91	105	106	120	121	135	136	150	151	165	166	180
1	PSA	-----	GHSLFHP	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	DAVKVMDLPTQ-BEA	147	
2	hK2	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	147	
3	hK1	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	147	
4	HSCCE	-----	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	DAVKVMDLPTQ-BEA	148	
5	5yme	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	148	
6	6 K1K-16	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	148	
7	7 TLSP	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	148	
8	8 K1K-14	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	148	
9	9 NES1	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	148	
10	10 K1K-15	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	148	
11	neuropsin	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	148	
12	protease	-----	LTPAALCKKNSQVWL	GHNLPEF	EDTQGV	FGVSHSPFHPLYDS	-----	LKLNRFIPDQSDS	DLMLRLSEPAE-LT	148	

# FIGURE 43 (CONT'D)

181	195	196	210	211	225	226	240	241	255	256	270
1 PSA	LGPTCYASGWSIEP	EEFLTPKKLQCVDH	VISNDVCAQVHPQV	TKFMLCAGRWTKGKS	TCGSDSGGPLVNCNV	LQGITSMG	-SEPCAL	236			
2 hK2	LGPTCYASGWSIEP	EEFLTPKKLQCVDH	LISNDMCARAYSEKV	TEFMLCAGLWTKGND	TCGSDSGGPLVNCNV	LQGITSMG	-PEPCAL	236			
3 hK1	VGSTCTLASGWSIEP	ENFSPDIQCVDLK	LSPDCTCKVYKDL	ENSMCLAGLPESSKN	ACNDSGGPLVNCNV	LQGITSMG	-TFPCGQ	237			
4 HSCCE	PFTTCTVSGWGTTS	PDVTFPSIDLMCVDLK	LSPDCTCKVYKDL	ENSMCLAGLPESSKN	ACNDSGGPLVNCNV	LQGITSMG	-TFPCGQ	237			
5 zyme	NTTSCSHLWGKTD	--GDFPDTIQCAVH	LVSRECEHAYFGI	TDNMFCAGDEKVGVD	SCQSDSGGPLVNCNV	LQGITSMG	-TFPCGQ	228			
6 KLK-L6	AGTSCSLISGWSITS	PLARPHTLRCANIT	LISPEVCORAYPRTI	TPSNVCAGVPOQSKD	SCQSDSGGPLVNCNV	LQGITSMG	-NIFCGS	220			
7 TLSP	EGTTCRSVSGWGTTS	POVNPYKTLQCANIT	LISPEVCORAYPRTI	TPSNVCAGVPOQSKD	SCQSDSGGPLVNCNV	LQGITSMG	-NIFCGS	227			
8 KLK-L4	PGDCCVACWGTGTA	RAVKNYKGLTCSSTJ	LISPEVCORAYPRTI	TPSNVCAGVPOQSKD	SCQSDSGGPLVNCNV	LQGITSMG	-QDPCAL	226			
9 NES1	AGTTCRSVSGWGTTS	POVNPYKTLQCANIT	LISPEVCORAYPRTI	TPSNVCAGVPOQSKD	SCQSDSGGPLVNCNV	LQGITSMG	-YFPCGQ	241			
10 KLK-L5	AGTTCRSVSGWGTTS	POVNPYKTLQCANIT	LISPEVCORAYPRTI	TPSNVCAGVPOQSKD	SCQSDSGGPLVNCNV	LQGITSMG	-YFPCGQ	252			
11 neutropsin	PGQCTVYSGWGTTS	PRNFPDTLNCAYR	IFPQKCEDATPQOI	TDNMVCAGSKR-GAD	TCQSDSGGPLVNCNV	LQGITSMG	-SDPCGR	224			
12 prostate	AGNSCLVUSWGLLAN	--GRMPTVLQCVNVS	VVSEVCSKLYDPLV	HPSMFCAGGGHDKD	SCNDSGGPLVNCNV	LQGITSMG	-KAPCGQ	230			
271	285	286	300	301	315	316	330	331	345	346	360
1 PSA	PERPSLYTKVHYR	WIKDTIVAMP	-----	261							
2 hK2	PEKPAVYTKVHYR	WIKDTIAANP	-----	261							
3 hK1	PNKPSAVRVLSTYK	WIEDTIAENS	-----	262							
4 HSCCE	PNDEGYVTVCKSTK	WINDTKKRR	-----	253							
5 zyme	KEKPGVYTVCKSTK	WIKETIAQK	-----	244							
6 KLK-L6	PGYPGVYTVCKSTK	WIKETIAQK	-----	251							
7 TLSP	TRKPGVYTVCKSTK	WIKETIAQK	-----	250							
8 KLK-L4	PDREGYVTVCKSTK	WIKETIAQK	-----	277							
9 NES1	AQHEPVYTVCKSTK	WIKETIAQK	-----	276							
10 KLK-L5	DGIEGYVTVCKSTK	WIKETIAQK	-----	248							
11 neutropsin	SDKEGYVTVCKSTK	WIKETIAQK	-----	260							
12 prostate	GVGPGVYTVCKSTK	WIKETIAQK	-----	254							



**FIGURE 44**

## Sequence Listing

SEQ.ID. NO. 1

KLK-L1 na

1 tatctcatga gagagaataa gaacatgaaa agagaaagaa tgagagagag  
agagagaaaag  
61 aaaaaggaga gtggagtcta ggatctgggc aggggtctcc tccctgggtc  
cctagaccct  
121 gctgccagcc cctctcgggc ccccaaccac tgcctgtgta gagtgtaggc  
agcctgagag  
181 agttgagctg gaagtttgca gcacctgacc cctggaacac atccctggg  
ggcaggccag  
241 cccaggctga ggatgcttat aagcccaag gaggccctg cgagggcagc  
aggtctgggc  
301 tcagcccagc agtgaatcc aggagccag aggtggccgg gtaagaggcc  
tggtgggtccc  
361 ccactaaaag cctgcagtgt tcatgatcca actctcccta cagctccatg  
tcgctggatt  
421 ctacgcctct gtgccttctg tctccacatc tctctagaca gatctctcac  
tgtctctagt  
481 taggagtcac tgtctctagt taggggtctc tctgtctctc tgaatctata  
tctccatgtc  
541 taactctcag actgtctctg aggatctctc tcaagcactc tgtctctccg  
gctctgattc  
601 tctgtgtgtc tccctccat gcttgtttgt ggggtggctag acaccatctc  
tccccattca  
661 cagatggcta gatgctttct ctaaacttcc ctttctacct agttctctct  
ctctctcttt  
721 tcccatctct ctctctcttt ttctctctca gtctctaaat ctgtctctct  
aggttctggg  
781 tccatggatg ggagaggggg tagatggtct aggtctcttc ctacctata  
acgtcccaga  
841 gggaagaaaag ggaggggaca agagagggat ggagagactt gggctgaaga  
tccccagaca  
901 cggetaagtc tcagtcctca tccccagggt ctgacgtgat ggccacagca  
ggaaatccct  
961 ggggctgggt cctggggtag ctcatccttg gtgtcgcagg tatctgagta  
tggtgtgtgt  
1021 tgtctgtccg tgcttggggg cacagtgttt gttaatgttc aggtgtgact  
cagtgtctc  
1081 ttgcttgtga ctgcaaagct gcctgtgaga cggtagccgt tttatccgtcc  
gccatggctg  
1141 tgccctgca-actccttgta tcgtggtaaa tttgtgtgtg gcagtgtgcc  
tgggtgtgtg  
1201 gttgtacctg tgagactctg acagtttgtg cctctgaata tctgggtggag  
tgacaacagt  
1261 gtaatgatga tatggggaca ggggaagccg aggtgtcagg agattgtgct  
tccctggggcg  
1321 tgatccattg ctgggaatct gtgcctgctt cctgggtctt cagtccctgag  
atccctctct  
1381 cccatcccca aggaactcac ctacacaggac tataaaacgg tgttttgggtg  
tgcattgggt  
1441 tgtggcttgg tgtgactgtg ggcaaggctg ggagaggata ggagtgtact  
ggcgacaggac  
1501 cgaactcttg agcatcagtc tgcgcagaca agtgaccga tccctgtctc  
cagcaacaac  
1561 tccacccct gagctttaat tcaccccgaa ggacccgac ctaccgctat  
gagcctagac

1621 tctctgtgtg aacccctect gaccgtggct ttgcaccgcg atggcaccag  
 tctcacctcc  
 1681 agagctcacc ccagagccct gactccgcc cagaagccct ggtcccaact  
 tctgagactg  
 1741 cctctagcca taaccagctt ctggaagcct tgatggcgcc cctgcgctgt  
 aacccaacc  
 1801 ctaggagcac tgatcccgcc ttctcagccc acccccatgc cctgactctc  
 ctcccaggag  
 1861 ccctgactac cctgaatecc tgaccaggct cctgcaccgt gatcacccgc  
 cctgggagcc  
 1921 ctaggcctat atcctggacc agccctgaa gctccgatca tgaccctgc  
 accataaccc  
 1981 cacccccagg agccctgggt ccgccccctg ggcggccccc cagccctgac  
 tcggccccc  
 2041 aagagtccct actgctcctg aagccctgac cagcccccgt ctccgtaacc  
 cctcccccaa  
 2101 gagccctggg ccgcctctct gagcccgctt ccagccctga ctccgccccg  
 aggagccctg  
 2161 actgctcctg aacctctgac cagcccccgt ctccgtaagc ccaccccag  
 gaaccctggg  
 2221 cccgcctect ggtcccgatc ccatecctga ctccgcccctc aggatctctc  
 gtctctggta  
 2281 gctgcagcca aatcataaac ggcgaggact gcagcccgca ctgcagccc  
 tggcaggcgg  
 2341 cactggtcat ggaaacgaa ttgttctgct cgggcgtcct ggtgcatccg  
 cagtgggtgc  
 2401 tgtcagccgc acactgtttc cagaagttag tgacagagga gggggagtgg  
 gcaggccctg  
 2461 ggtccggggg cggggcctaa tatcaggctc atcttggggg gctcaggggg  
 aaacagcgg  
 2521 gaaggctctg ggaggaggac ggaatgagcc tggatccggg gagccagag  
 ggaaggcctg  
 2581 ggaggcggga atcttgcttc ggaaggactc agagagccct gacttgaat  
 ctacgcccag  
 2641 tgctgagtct ctagtgaact aaggcaagtt ctgtccctg aatttttctg  
 aatgaggatt  
 2701 tgagaccatg gttaagttagc tttaggggtg tttagcgaag aggggtgggt  
 tggggttagg  
 2761 agatggggat ggggaatggg ttgaagatga gaatggaggt aggatgtag  
 ttgccacaaa  
 2821 actgacctgc cctccgtggc ccacagctcc tacaccatcg ggctgggcct  
 gcacagtctt  
 2881 gaggccgacc aagagccagg gagccagatg gtggaggcca gcctctccgt  
 acggcaccca  
 2941 gagtacaaca gacccttgct cgctaacgac ctcatgtcca tcaagtggga  
 cgaatccgtg  
 3001 tccgagtctg acaccatccg gagcatcagc attgcttcgc agtgccttac  
 cgcggggaac  
 3061 tcttgctctg tttctggctg ggtctgctg gcgaacgggt agctcacggg  
 tgtgtgtctg  
 3121 cctcttcaa ggaggctctc tgcccagtcg cgggggctga ccagagctc  
 tgcgtcccag  
 3181 gcagaatgcc taccgtgctg cagtgcgtga acgtgtcgtt ggtgtctgag  
 gaggtctgca  
 3241 gtaagctcta tgaccgctg taccacccca gcatgttctg cgcggcgga  
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 3301 agaaggactc ctgcaacgtg agagagggga aaggggaggg caggcgactc  
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 tggagagaca

3421 cacagggaga cagtgacaac tagagagaga aactgagaga aacagagaaa  
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 gaaacacaca  
 3541 cacatagaaa tgcagttgac cttccaacag catggggcct gagggcgggtg  
 acctccaccc  
 3601 aatagaaaa cctcttataa cttttgactc cccaaaaacc tgactagaaa  
 tagcctaactg  
 3661 ttgacgggga gccttaccaa taacataaat agtcgattta tgcatacggtt  
 ttatgcattc  
 3721 atgatataacc tttgttgga ttttttgata tttctaagct acacagttcg  
 tctgtgaatt  
 3781 tttttaaat gttgcaactc tcctaaaatt tttctgatgt gtttattgaa  
 aaatccaag  
 3841 tataagtga cttgtgcagt tcaaacacagg gttgttcaag ggtaactgt  
 gtaccagag  
 3901 ggaacacagt acacagattc atagagggtg aacacgaaga gaaacaggaa  
 aatcaagac  
 3961 tctacaaga ggctgggagc ggtggctcat gcctgtaac ccagcacttt  
 gggaggcag  
 4021 gcaggcagat cacttgaggt aaggagtta agaccagcct ggccaaaatg  
 gtgaaatcct  
 4081 gtctgtacta aaaatacaaa agttagctgg atatggtggc aggcgcctgt  
 aatcccagct  
 4141 acttgggagg ctgaggcagg agaattgctt gaatatggga ggagaggtt  
 gaagtgaatt  
 4201 gagatcacac cactatactc cagctggggc aacagagtaa gactctgtct  
 caaaaaaaa  
 4261 aaaaaaaaag actttacaaa gagatgcaga gacactgaga cagataaaca  
 agccacaag  
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 agaagcagcc  
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SEQ.ID. NO. 2

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 SESDTIR SISIASQCPTAGNSCLVSGWGL LANGE LTRMPTVLQCVNVSVVSE  
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SEQ.ID. NO. 3

KLK-L1

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SEQ.ID. NO. 4

Figure 4 Sequence

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TTTCGGAAAA GCCCCGTGTG GCCAAGTTGG CGTGCCAGGT  
GCCTACACCA ACCTCTGCAA  
ATTCACTGAG TGGATAGAGA AAACCGTCCA GGCCAGTTAA  
CTCTGGGGAC TGGGAACCCA  
TGAAATTGAC CCCCAAATAC ATCCTGCGGA AGGAATTC

SEQ.ID. NO. 5

Table 8 Sequence

TGACCCGCTGTACCACCCCA

SEQ.ID. NO. 6

Table 8 Sequence

GAATTCCTCCGCAGGATGT

SEQ.ID. NO. 7

Table 8 Sequence

GGTGATCTGCGCCCTGGTCCT

SEQ.ID. NO. 8

Table 8 Sequence

AGGTGTCCGGTGGAGGTGGCA

SEQ.ID. NO. 9

Table 8 Sequence

TGCGCAAGTTCACCTCA

SEQ.ID. NO. 10

Table 8 Sequence

CCCTCTCCCTTACTTCATCC

SEQ.ID. NO. 11

Table 8 Sequence

ACAATGAGCTGCGTGTGGCT

SEQ.ID. NO. 12

Table 8 Sequence

TCTCCTTAATGTCACGCACGA

SEQ.ID. NO. 13

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SEQ.ID. NO. 15

Table 11

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SEQ.ID. NO. 16

Table 11

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SEQ.ID. NO. 17

Table 11

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SEQ.ID. NO. 18

Table 11

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SEQ.ID. NO. 19

Table 11

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SEQ.ID. NO. 20

Table 11

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SEQ.ID. NO. 21

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SEQ. ID. NO. 23

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SEQ. ID. NO. 24

Table 13

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SEQ. ID. NO. 25

Table 13

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SEQ. ID. NO. 26

Table 13

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SEQ. ID. NO. 27

Table 14

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SEQ. ID. NO. 28

TABLE 14

GCCACTGCCTGATGGAGACTG

SEQ. ID. NO. 29

TABLE 14

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TABLE 14

CTTCGGCCTCTCTTGGTCTT

SEQ. ID. NO. 31

TABLE 14

GGGTCAGAGCTGCAGAGAAG

SEQ. ID. NO. 32

TABLE 14

GGGCCTGTCGTCTGCAATGG

SEQ. ID. NO. 33

TABLE 14

ATGGCCACAGCAGGAAATCC

SEQ. ID. NO. 34

TABLE 14

GGTCACTTGTCTGCGCAGAC

SEQ. ID. NO. 35

TABLE 14

CCCAACCCTGTGTTTTTCTC

SEQ. ID. NO. 36

GGCCCTCCTCCCTCAGA

SEQ. ID. NO. 37

TABLE 14

ATCCCTCCATTCCTCTTT

SEQ. ID. NO. 38

TABLE 14

CACATACAATTCTCTGGTTC

SEQ. ID. NO. 39

TABLE 14

AGTGACACTGTCTCAGAATT

SEQ. ID. NO. 40

TABLE 14

CCCCAATCTCACCAGTGCAC

SEQ. ID. NO. 41

TABLE 14

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SEQ. ID. NO. 42

TABLE 14

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SEQ. ID. NO. 43

KLK-L4 NA

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SEQ.ID.NO. 45

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SEQ.ID.NO. 46

Table 15

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SEQ.ID.NO. 47

Table 15

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SEQ.ID.NO. 48

Table 15

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SEQ.ID.NO. 49

Table 15

AGGCTGCCCTACTAGTGCAA

SEQ.ID.NO. 50

Table 15

ATATTGCCTAGGTGGATGTG

SEQ.ID.NO. 51

Table 15

AAGACTTCAAGGAGCCAAGC

SEQ.ID.NO. 52

Table 15

GACCC TTCACCTCCCAAAAT

SEQ.ID.NO. 53

Table 15

CTAGTGATCGCCTCCCTGAC

SEQ.ID.NO. 54

Table 15

GGTGATCTGCGCCCTGGTCCT

SEQ.ID.NO. 55

Table 15

AGGTGTCCGGTGGAGGTGGCA

SEQ.ID.NO. 56

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SEQ.ID.NO. 57

KLK-L5 AA

LSQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGVLIDHRWVLTAAHCSG  
 SRYWVRLGEHSLSQLDWTEQIRHSQFVTHPGYLGASTSHEHDLRLRLRLP  
 VRVTSSVQPLPLPNDCATAGTECHVSGWGITNHPNPPFDLLQLCLNSIVSHA  
 TCHGVYPGRITSNMVCAGGVPGQDACQ

SEQ.ID.NO. 58

KLK-L5 AA – alternatively spliced

MGLSIFLLCVLGLSQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGVLID  
 HRWVLTAAHCSGRPIGSAVPVQPLHRLPCHLPWCVSRENHEQHGVCRRRPG  
 AGCLPG

SEQ.ID.NO. 59

KLK-L5 AA – alternatively spliced

MGLSIFLLLCVGLGSLQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGVLID  
HRWVLTAAHCSGSRYWVRLGEHLSQLDWTEQIRHSGFSVTHPGYLGASTS  
HEHDLRLRLRLPVRVTSSVQPLPLPNDCATAGTECHVSGWGITNHPNPFDP  
LLQCLNLSIVSHATCHGVYPGRITSNMVCAGGVPQDACQGDSSGGPLVCGG  
VLQGLVSWGSGVPCGQDGIPGVYTYICNSTLVGLGTSWNFNSCQPF

SEQ.ID.NO. 60

KLK-L5-AA

MGLSIFLLLCVGLGSLQAATPKIFNGTECGRNSQPWQVGLFEGTSLRCGGVLID  
HRWVLTAAHCSGSRYWVRLGEHLSQLDWTEQIRHSGFSVTHPGYLGASTS  
HEHDLRLRLRLPVRVTSSVQPLPLPNDCATAGTECHVSGWGITNHPNPFDP  
LLQCLNLSIVSHATCHGVYPGRITSNMVCAGGVPQDACQGDSSGGPLVCGG  
VLQGLVSWGSGVPCGQDGIPGVYTYICKYVDWIRMIMRNN

SEQ.ID.NO. 61

Table 17

TCAGCCAGGCAGCCACACCG

SEQ.ID.NO. 62

Table 17

TTGGTGATGCCCCAGCCTGA

SEQ.ID.NO. 63

Table 17

CCACACCGAAGATTTTCAAT

SEQ.ID.NO. 64

Table 17

GCCCCCTCCTTCATTTATA

SEQ.ID.NO. 65

## KLK-L6 NA

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 gtctctgacg  
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 gtggatcatt  
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 361 ctgcactcca gccggggcta aagagtgaga ctctgtctca aaaaaaaaaa  
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 421 aaaaaagaa aaaaaataa aataataaa taaataaat aaatttaaa  
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 481 aaagaggggg ttctgtgtt gatgccgagc ctgaaccaag gcagaggagg  
 ccgggaaggc  
 541 ttcccaaggc ctctagctca aagcaggagg gcccatagtt aaacagaaac  
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 2221 ccttggttct ctgactccac cactagatcc accacctcca gcaactggga  
 accctccct  
 2281 gccaccctg cctggggctc cctcccagg atctcttcta gattatagca  
 tcttccctgg  
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 gaggggatgg  
 2401 gatcaggagg tcttggaaatg ggaactaggc aataaaaaaa aaaaaatgtc  
 agaagcaggg  
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 gggacctgtg  
 2581 cctgtctcca catctctcca cctgcccgc gcagagcctg caggccccgc  
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 tataagccat  
 4861 atctatgcca taacaggctc agtctagaac ctgagagatca aagctcaggc  
 cagagtctag  
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 ctagaanaacg

5341 aatgggtgctt actccggaat atcagttccg atttagagcc tagactcata  
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 gctctcccaa  
 5461 tcatcacctt ctcccaagc caggagctgg agcccagccc aagagcggaa  
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 caactccggg  
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 6481 ctctgcaatg cgtgaacatc aacatctccc cggatgaggt gtgccagaag  
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7141 ctaactgtcc accagtgtct ctgctgtct cctgtgtct gcttctcggg  
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8041 ttagtgtctc taaaaaccga ataataatg acaataaaaa tggaatcatc  
ctaaattgta  
8101 ttcattcatc catgtgttta ctttttattt ttgagacaa ggtcttgctc  
agtctcctgg  
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tccagtgtac  
8221 ctctactc agctcccca gtagctgga ccacaggtc ccgtccat gccccgtac

SEQ.ID.NO. 66

KLK-L6 AA

MTQSQEDENKIIGGHTCTRSSQPWQAALLAGPRRRFLCGGALLSGQWVITAA  
HCGRPILQVALGKHNLRRWEATQQVLRVVRQVTHPNYNSRTHDNDMLLQL  
QQPARIGRAVRPIEVTQACASPGTSCRVSQWGTISSPIARYPASLQCVNINISPD  
EVCQKAYPRITITPGMVCAGVPQGGKDSQGDSSGGLVCRGQLQGLVSWGM  
ERCALPGYPGVYTNLCKYRSWIEETMRDK

SEQ.ID.NO. 67

KLK-L6 AA

MFLLLTALQVLAIAMTQSQEDENKIIGGHTCTRSSQPWQAALLA  
GPRRRFLCGGALLSGQWVITAAHCGRPILQVALGKHNLRRWEATQQVLRVV  
RQVTHPNYNSRTHDNDMLLQLQQPARIGRAVRPIEVTQACASPGTSCRVSQ  
WGTISSPIARYPASLQCVNINISPD EVCQKAYPRITITPGMVCAGVPQGGKDS

QGDSSGGLVCRGQLQGLVSWGMECALPGYPGVYTNLCKYRSWIEETMRD  
K

SEQ.ID.NO. 68

Figure 9

prostate

MATAGNPWGWFLGYLILGVAGSLVSGSCSQIINGEDCSPHSQPWQAALVME  
NELFCSGVLVHPQWVLSAAHCFQNSYTIGLGLHSLEADQEPGSMVEASLSV  
RHPYENRPLLANDLMLIKLDESVSSEDTIRISIASQCPTAGNSCLVSWGGLA  
NGRMPITVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDCSCNGDSG  
GPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTIEWIEK

SEQ.ID.NO. 69

Figure 9

EMSP

MATAGNPWGWFLGYLILGVAGSLVSGEMSPSCSQIINGEDCSPHSQPWQAAL  
VMENELFCSGVLVHPQWVLSAAHCFQNSYTIGLGLHSLEADQEPGSMVEAS  
LSVRHPEYNRPLLANDLMLIKLDESVSSEDTIRISIASQCPTAGNSCLVSWG  
LLANGRMPTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQKDCSCNG  
DSGGPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTIEWIEK 249

SEQ.ID.NO. 70

Figure 9

KLK-L2

MATARPPWMWVLCALITALLGVTEHVLANNNDVSCDHPSTNVPSGSNQDLG  
AGAGEDARSDDSSRIINGSDCDMHTQPWQAALLLRPNQLYCGAVLVHPQW  
LLTAAHCRKKVFRVRLGHYSLSPVYESGQMQFQGVKSIPHPGYSHPGHSNDL  
MLIKLNRIRPTKDVPRINVS SHCPSAGTKCLVSGWGTTKSPQVHFPPKVLQCL  
NISVLQKRCEDA YPRQIDDTMFCAGDKAGRDSCQGDSSGPPVVCNSGLQGL  
VSWGDIYPCARPNRPGVYTNLCKFTKWIQE

SEQ.ID.NO. 71

Figure 9

zyme

MKKLMVVLSLIAAAWAEQNKL VHGGPCDKTSHPYQAALYTSGHLLCGGV

LIHPLWVLTAAHCKKPNLQVFLGKHNLRORESSQEQQSSVVRAVIHPDYDAAS  
HDQDIMLLRLARPAKLSLIQPLPLERDCSANTTSCHILGWGKTADGDFPDTI  
QCA YIHLVSREECEHAYPGQITQNMLCAGDEKYGKDSCQGDSSGGPLVCGDH  
LRGLVSWGNI PCGSKKEKPGVYTNVCR YTNWIQK

SEQ.ID.NO. 72

Figure 9

neuropsin

MGRPRPRAAKTWMFLLLLGGAWAGHSRAQEDKVLGGHECQPHSQPWQAA  
LFQGGQQLLCGGVLVGGNWVLTAAHCKKPKYTVRLGDHSLQNKDGPEQEI PV  
VQSI PHPCYNSSDVEDHNHDLMLLQLRDQASLGSKVKPISLADHCTQPGQKC  
TVSGWGT VTS PRENFPDTLNCAEVKIFPQKKCEDAYPGQITDGMVCAGSSKG  
ADTCQGDSSGGPLVCDGALQGITSWGSDPCGRSDKPGVYTNICRYLDWIKKTL  
SPMRILQLILLALATGLVG

SEQ.ID.NO. 73

Figure 9

TLSP

GETRIIKGFECKPHSQPWQAALFEKTRLLCGATLIAPRWLLTAAHCLKPRYIV  
HLGQHNLQKEEGCEQTRTATESFHPGFNNSLPNKDHRNDIMLVKMASPVSI  
TWA VRPLTLSSRCVTAGTSCLSIGWGSTSSPQLRLPHTLRCANTTIEHQKCEN  
AYPGNITD TMVCASVQEGGKDS CQGDSSGGPLVCNQSLQGIISWGQDPCA ITR  
KPGVYTKVCKYVDW IQE

SEQ.ID.NO. 74

Figure 9

PSA

MWVPVVFLLT SVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG  
GVLVHPQWVLTAAHCIRNKS VILLGRHSLFHPEDTGQVFQVSHSFPHPLYDM  
SLLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTTCYAS  
GWGSIEPEEF LTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGK  
STCSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKD

SEQ.ID.NO. 75

Figure 9

KLK2

MWDLVLIALSVGCTGAVPLIQSRIVGGWECEKHSQPWQVAVYSHGWAHCG  
 GVLVHPQWVLTAAHCLKKNSQVWLGRHNLFEEDTGQRVPVSHSFPHPLYN  
 MSLKHKQLRPDESSHDLMLLRLSEPAKITDVVKVLGLPTQEPALGTTTCYAS  
 GWGSEIEPEEFLRPRSLQCVSLHLLSNDMCARAYSEKVTTEFMLCAGLWTGGKD  
 TCGGDSGGPLVNCVGLQGITSWGPEPCALPEKPAVYTKVVHYRKWIKD

SEQ.ID.NO. 76

Figure 9

KLK1

MWFLVLCLALSLGGTGAAPPIQSRIVGGWECEQHSQPWQAALYHFSTFQCGG  
 ILVHRQWVLTAAHCISDNQYLWLGRHNLFDDENTAQFVHVSESFPHPGFNMS  
 LLENHTRQADEYSHDLMLLRLTEPADTTIDAVKVVVELTPEEPEVGSTCLASG  
 WGSIEPENFSFPDDLQCVDLKILPNDECKAHVQKVTFMLCVGHLEGGKDT  
 CVGDSGGPLMCDGVLQGVTSWGYVPCGTPNPKPSVAVRVLVSYVKWIED

SEQ.ID.NO. 77

Figure 9

trypsinogen

MNPLLLTFVAAALAAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGGSLI  
 NEQWVVSAGHCYKSRIVRLGEHNIEVLEGNEQFINAAKIRHPQYDRKTLNN  
 DIMLIKLSRAVINARVSTISLPTAPPATGTCKLISGWGNTASSGADYPDELQC  
 LDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSQCQGDSSGGPVVNCGQLQ  
 GVVSWG-DGCAQKNKPGVYTKVYNVVKWIKN

SEQ.ID.NO. 78

Figure 17, 27, 36, 43

PSA

MWVPVVFVLTLSVTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCG  
 GVLVHPQWVLTAAHCIRNKSIVLLGRHSLFHPEDTGQVFQVSHSFPHPLYDM  
 SLLKNRFLRPDDSSHDLMLLRLSEPAELTDVAVKVMIDLPTQEPALGTTTCYAS  
 GWGSEIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQVKTFMLCAGRWTGGK  
 STCSGDSGGPLVCNCGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDTIVA  
 NP

SEQ.ID.NO. 79

Figure 17, 27, 36, 43

KLK2

MWDLVLSIALSVGCTGAVPLIQSRIVGGWECEKHSQPWQVAVYSHGWAHCG  
 GVLVHPQWVLTAAHCLKKNSQVWLGRHNLFEPEDTGQRVPVSHSFPHPLYN  
 MSLLKHQSLRPDESSHDLMMLRLSEPAKITDVVKVLGLPTQEPALGTTTCYAS  
 GWGSIPEEFELRPRSLQCVSLLHLLSNDMCARAYSEKVTEFMLCAGLWTGGKD  
 TCGGDSGGPLVNCNGVLQGITSWGPEPCALPEKPAVYTKVVHYRKWIKDTIAA  
 NP

SEQ.ID.NO. 80

Figure 17, 27, 36, 43

KLK1

MWFLVLCLALSLGGTGAAPPIQSRIVGGWECEQHSQPWQAALYHFSTFQCCG  
 ILVHRQWVLTAAHCISDNYQLWLGRHNLFDDENTAQFVHVSESFPHPGFNMS  
 LLENHTRQADEDYSHDLMLRLTEPADTITDAVKVVELPTEEPVGSTCLASG  
 WGSIEPENFSFPDDLQCVDLKLPNDECKKAHVQKVTFMCLCVGHLEGGKDT  
 CVGDSGGPLMCDGVLQGVTSWGYVPCGTPNKPVSVAVRVLSYVKWIEDTIAE  
 NS

SEQ.ID.NO. 81

Figure 17, 27, 36, 43

protease

MATAGNPWGWFLGYLILGVAGSLVSGSCSQIHNGEDCSPHSQPWQAALVME  
 NELFCSGVLVHPQWVLSAAHCFQNSYTIGLGLHSLEADQEPGSGQMVEASLSV  
 RHPEYNRPLLANDLMLIKLDESVS-  
 ESDTIRISIASQCPTAGNSCLVSGWGGLANG—  
 RMPTVLQCVNVSVVSEEVCSKLYDPLYHPSMFACAGGGHDQKDCNGDSGGP  
 LICNGYLQGLVSFGKAPCGQVGPVGYTTLCKFTEWIEKTVQAS

SEQ.ID.NO. 82

Figure 17, 27

trypsinogen

MNPLLLITFVAAALAAPFDDDDKIVGGYNCEENSVPYQVSLNSGYHFCGSLI  
 NEQWVVSAGHCYKSRIQVRLGEHNIEVLEGNEQFINAAKIIRHPQYDRKTLNN  
 DIMLIKLSSRAVINARVSTISLPTAPPATGKCLISGWGNTASSGADYPDELQC  
 LDAPVLSQAKCEASYPGKITSNMFCVGFLEGGKDSQCQDSGGPVVNCNGQLQ  
 GVVSWG-DGCAQKNKPGVYTKVYNVYKWKIKNTIAANS

SEQ.ID.NO. 83

Figure 17, 27, 36, 43

neuropsin

MGRPRPRAAKTWMFLLLLGGAWAGHSRAQEDKVLGGHECQPHSQPWQAA  
LFQGGQQLLCGGVVLVGGNWVLTAACHCKPKPYTVRLGDHSLQNKDGPQEIPV  
VQSIHPHCYNSSDVEDHNHDLMLLQLRDQASLGSKVKPISLADHCTQPGQKC  
TVSGWGTVTSPRENFPDTLNCAEVKIFPQKKCEDA YPGQITDGMVCAGSSKG  
ADTCQGDGGPLVCDGALQGITSWGS DPCGRSDKPGVYTNICRYLDWIKKIIG  
SKG

SEQ.ID.NO. 84

Figure 17, 27, 36, 43

zyme

MKKLMVVLSLIAAAWAEQNKL VHGGPCDKTSHPHYAAL YTSGHLLCGGV  
LIHPLWVLTAACHCKPNLQVFLGKHNL RQRESSQEQQSSVVRAVIHPDYDAAS  
HDQDIMLLRLARPAKLSLIQPLPLERDCSANTT SCHILGWGKTADGDFPDTI  
QCA YIHLVSREECEHA YPGQITQNMLCAGDEKYGKDSCQGDGGPLVCGDH  
LRGLVSWGNI PCGSKKEKPGVYT NVCRYTNWIKTIQAK

SEQ.ID.NO. 85

Figure 27

EMSP

MATAGNPWGWFLGYLILGVAGSLVSGEMSPSCSQIINGEDCSPHSQPWQAAAL  
VMENELFCSGVLVHPQWVLSAAHCFQNS YTI GLGLHSLEADQEPG SQMVEAS  
LSVRHPEYNRPLLANDMLIKLDESVS ESDTIRSIASQCPTAGNSCLVSGWG  
LLANGRMPTVLQCVNVSVVSEEVCSKLYDPLYHPSMFCAGGGHDQK DSCNG  
DSGGPLICNGYLQGLVSFGKAPCGQVGVPGVYTNLCKFTEWIEKTVQAS

SEQ.ID.NO. 86

Figure 27

TLSP

MRI-LQLILLALATGLVGGETRIIKGFECKPHSQPWQAAALFEKTRLLC  
GATLIAPRWLLTAACHCLKPRYIVHLGQHNLQKEEGCEQTRTATESFPHPGFNN  
SLPNKDHRNDIMLVKMASPV SITWAVRPLTLSSRCVTAGT SCLISGWGSTSSP  
QLRLPHTLRCANTITIEHQKCENAYPGNITDTMVCASVQEGGKDSCQGDGGP  
LVCNQLSQGIISWGQDPCAITRKPGVYTKVCKYVDWIQETMKNN

SEQ.ID.NO. 87

Figure 27

HSCEE

MARSLLLPLQILLLSLALETAGEEAQGDKIIDGAPCARGSHPWQVALLSGNQL  
HCHSCCEGGVLVNERWVLTAAHCKMNEYTVHLGSDTLGDRRAQRIKASKSF  
RHPGYSTQTHVNDLMLVKLNSQARLSSMVKKVRLPSRCEPPGTTCTVSGWG  
TTTSPDVTFPDLMCVDVKLISPQDCTKVYKDLENSMLCAGIPDSKKNACNG  
DSGGPLVCRGTLQGLVS WGTFFCGQPNDPGVYTVCKFTKWINDTMKKHR

SEQ.ID.NO. 88

Figure 27

NES1

MRAPHLHLSAASGARALAKLLPLLMAQLWAAEAALLPQNDTRLDPEAYGAP  
CARG SQPWQVSLFNLSFH CAGVLVDQSWVLTAAHCGNKPLWARVGDDH  
LL-LLQG-EQLRRTT RSVVHPKYHQSGPI LPRRTDEHDLML LKLARPVV-  
PGPRVR ALQLPYR-CAQPGDQ CQVAGWGTTAARRVK YNKGLTCSSTITLSP  
KECEVFYPGVVTNNM ICAGLDR-GQDPCQS DSGGPLVCDETLQGI LSWG-  
VYPCGSAQHPAVYTQICKYMSWINK VIRSN

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/CA 00/00258

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/57 C12N9/64 C12N15/63 C07K16/40 C12Q1/68  
G01N33/53 A61K39/395 A61K48/00 A61K38/48 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K C12Q G01N A61K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.P. SIMMER ET AL.: "Purification, characterization, and cloning of Enamel Matrix Serine Proteinase 1" JOURNAL OF DENTAL RESEARCH, vol. 77, no. 2, February 1998 (1998-02), pages 377-386, XP000910876 page 380, right-hand column, paragraph 2 -page 384, right-hand column, paragraph 1; figure 6 ---	1-8, 14-31
A	P.H.J. RIEGMAN ET AL.: "Characterization of the human Kallikrein locus" GENOMICS, vol. 14, 1992, pages 6-11, XP002058183 cited in the application the whole document --- -/--	1-8, 14-31

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance  
 "E" earlier document but published on or after the international filing date  
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
 "O" document referring to an oral disclosure, use, exhibition or other means  
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"S" document member of the same patent family

Date of the actual completion of the international search

6 July 2000

Date of mailing of the international search report

18. 10. 00

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C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LYNETTE J. SCHEDLICH ET AL.: "Primary structure of a human glandular Kallikrein gene" DNA, vol. 6, no. 5, 1987, pages 429-437, XP002044466 cited in the application the whole document ---	1-8, 14-31
A	P.H.J. RIEGMAN ET AL.: "Characterization of the prostate-specific gene: A novel human Kallikrein-like gene" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 159, no. 1, 28 February 1989 (1989-02-28), pages 95-102, XP002141957 ORLANDO, FL US cited in the application the whole document ---	1-8, 14-31
A	WO 98 20117 A (INCYTE PHARMACEUTICALS, INC.) 14 May 1998 (1998-05-14) page 10, line 25 -page 36, line 18 ---	1-8, 14-31
P,X	GEORGE M. YOUSEF ET AL.: "Protease/CLK-L1 is a new member of the human Kallikrein gene family, is expressed in prostate and breast tissues, and is hormonally regulated" CANCER RESEARCH, vol. 59, no. 17, 1 September 1999 (1999-09-01), pages 4252-4256, XP002141958 MD US the whole document ---	1-8, 14-31
P,X	PETER S. NELSON ET AL.: "Molecular cloning and characterization of prostate, an androgen-regulated serine protease with prostate-restricted expression" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 96, no. 6, 16 March 1999 (1999-03-16), pages 3114-3119, XP002141959 WASHINGTON US abstract page 3115, right-hand column, paragraph 3 -page 3116, right-hand column, paragraph 3 page 3117, right-hand column, paragraph 2 -page 3119, left-hand column, paragraph 2 ---	1-8, 14-31

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00258

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>STEPHENSON S A ET AL.: "Localization of a new prostate-specific antigen-related serine protease gene, KLK4, is evidence for an expanded human kallikrein gene family cluster on chromosome 19q13.3-13.4" JOURNAL OF BIOLOGICAL CHEMISTRY, [Online] vol. 274, no. 33, 13 August 1999 (1999-08-13), pages 23210-23214, XP002141960  MD US  Retrieved from the Internet:  &lt;URL:http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query_ol?uid=10438493&amp;form=6&amp;db=m&amp;Dopt=b&gt; [retrieved on 2000-05-17]  page 23211, left-hand column, paragraph 1  page 23211, right-hand column, paragraph 2 - paragraph 3; figure 1  page 23212, right-hand column, paragraph 2  -page 23214, left-hand column, last paragraph</p> <p style="text-align: center;">---</p>	1-8, 14-31
P,X	<p>DIAMANDIS E.P. ET AL: "The new human kallikrein gene family: Implications in carcinogenesis." TRENDS IN ENDOCRINOLOGY AND METABOLISM, (2000) 11/2 (54-60)., XP000910866  page 54, middle column, paragraph 2  -right-hand column, paragraph 2  page 56, right-hand column, paragraph 2  page 59, left-hand column, paragraph 2  -middle column, paragraph 2; figure 1; tables 1-3</p> <p style="text-align: center;">---</p>	1-8, 14-31
P,X	<p>GEORGE M. YOUSEF ET AL.: "Identification of novel human kallikrein-like genes on chromosome 19q13.3-q13.4" ANTICANCER RESEARCH, vol. 19, July 1999 (1999-07), pages 2343-2352, XP000911217  page 2845, right-hand column, paragraph 2  -page 2851, left-hand column, last paragraph; figure 1; table II</p> <p style="text-align: center;">---</p>	1-8, 14-31

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 00/00258

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>B.R. DUPONT ET AL.: "Assignment of serine protease 17 (PRSS17) to human chromosome bands 19q13.3-q13.4 by in situ hybridization" CYTOGENETICS AND CELL GENETICS, [Online] vol. 86, no. 3-4, 22 April 1999 (1999-04-22), pages 212-213, XP000911088 Retrieved from the Internet: &lt;URL:http://www3.ncbi.nlm.nih.gov/htbin-post/0mim/dispmin?603767#Referencel&gt; [retrieved on 2000-05-17] the whole document -----</p>	

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CA 00/00258**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 26 and 27 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds.
2. ☒ Claims Nos.: 26-29 partially  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
8 and partially claims 1-7 and 14-31

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 206

Continuation of Box 3.

Although claims 26 and 27 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compounds.

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Further defect(s) under Article 17(2)(a):

Continuation of Box 3.

Claims Nos.: 26-29 partially

Present claims 26-29 relate, partially, to a substance or compound identified according to the method of claim 22 or 23, defined by reference to a desirable characteristic or property, namely associating with, or modulating the biological activity of a protein of claims 8-13. The claims cover all substances or compounds having this characteristic or property, whereas the application does not provide support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for any specific example of such substances or compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved or a process for their preparation. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds other than identified according to the methods of claim 22 or 23.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

## FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

## 1. Claims: Claim 8 and partially claims 1-7 and 14-31

Kallikrein-like nucleic acid KLK-L1 and variants thereof; its regulatory sequence, vector and host cell comprising it and use thereof for preparing a protein; KLK-L1 protein and antibody against it; KLK-L1 probe; diagnostic and screening tests using KLK-L1 protein and nucleic acids; therapeutic uses thereof and their application in transgenic animals.

## 2. Claims: Claim 9 and partially claims 1-7 and 14-31

Kallikrein-like nucleic acid KLK-L2 and variants thereof; its regulatory sequence, vector and host cell comprising it and use thereof for preparing a protein; KLK-L2 protein and antibody against it; KLK-L2 probe; diagnostic and screening tests using KLK-L2 protein and nucleic acids; therapeutic uses thereof and their application in transgenic animals.

## 3. Claims: Claim 10 and partially claims 1-7 and 14-31

Kallikrein-like nucleic acid KLK-L3 and variants thereof; its regulatory sequence, vector and host cell comprising it and use thereof for preparing a protein; KLK-L3 protein and antibody against it; KLK-L3 probe; diagnostic and screening tests using KLK-L3 protein and nucleic acids; therapeutic uses thereof and their application in transgenic animals.

## 4. Claims: Claim 11 and partially claims 1-7 and 14-31

Kallikrein-like nucleic acid KLK-L4 and variants thereof; its regulatory sequence, vector and host cell comprising it and use thereof for preparing a protein; KLK-L4 protein and antibody against it; KLK-L4 probe; diagnostic and screening tests using KLK-L4 protein and nucleic acids; therapeutic uses thereof and their application in transgenic animals.

## 5. Claims: Claim 12 and partially claims 1-7 and 14-31

Kallikrein-like nucleic acid KLK-L5 and variants thereof; its regulatory sequence, vector and host cell comprising it and use thereof for preparing a protein; KLK-L5 protein and antibody against it; KLK-L5 probe; diagnostic and screening tests using KLK-L5 protein and nucleic acids; therapeutic uses thereof and their application in transgenic animals.

## 6. Claims: Claim 13 and partially claims 1-7 and 14-31

Kallikrein-like nucleic acid KLK-L6 and variants thereof; its regulatory sequence, vector and host cell comprising it and use thereof for preparing a protein; KLK-L6 protein and

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

antibody against it; KLK-L6 probe; diagnostic and screening tests using KLK-L6 protein and nucleic acids; therapeutic uses thereof and their application in transgenic animals.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9820117 A	14-05-1998	US 5786148 A AU 5165998 A EP 0941313 A US 5922321 A	28-07-1998 29-05-1998 15-09-1999 13-07-1999
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